DECLARATION OF CRAIG GERARD, MD, PH.D. UNDER 37 C.F.R. §1.132 Address to: Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Attorney Docket No.	RICE-050
	Confirmation No.	3065
	First Named Inventor	MACKAY, CHARLES REAY
	Application Number	10/584,480
	Filing Date	April 17, 2007
	Group Art Unit	1632
	Examiner Name	Wilson, Michael C
	Title: "TRANSGENIC NON-HUMAN MAMMAL COMPRISING A POLYNUCLEOTIDE ENCODING HUMAN OR HUMANIZED C5AR"	

Sir:

1. I, Craig Gerard, MD, Ph.D declare and say I am a resident of Chestnut Hill, Massachusetts.

2. I am an employee of the Children's Hospital Boston and Chief of the Division of Respiratory Diseases. Details of my career as well as publications may be found in my curriculum vitae (Exhibit 1).

3. I have been asked by FB Rice & Co, Patent Attorneys for G2 Inflammation Pty Ltd, to provide an independent opinion on the state of knowledge surrounding the C5a receptor in 2003 and on the invention described in US 10/584,480 (referred to below as the "Patent Application"). I have been asked in particular to comment on the obviousness rejections set out in the Office Action dated 8 January 2010 ("the Office Action") issued in connection with the Patent Application. I hold no equity position with either FB Rice & Co or G2 Inflammation Pty Ltd but I will be compensated for my time in preparing this Declaration.

4. I have read the Patent Application and have reviewed the claims that I understand are presently being considered by the United States Patent and Trademark Office. I understand that the claimed invention (referred to hereafter as the "Invention") relates to transgenic rodents which have been modified to express humanized C5aR. The importance of this invention is that it provides, for the first time, an *in vivo* model for identifying and testing for drugs that modulate signaling of human C5aR by C5a. A

number of inflammatory diseases are known to be the result of uncontrolled C5a/C5aR signaling and this *in vivo* model provides an important tool for identifying potential drugs to treat those inflammatory diseases.

5. I am very familiar with the technical field of the claimed invention. I have over 28 years of experience in research involving C5a and its receptor. My PhD thesis was on the purification and characterization of Porcine C5a (Craig Gerard, Ph.D. Thesis "Biochemical aspects of the Complement C5a Anaphylatoxin" 1981 University of California at San Diego). In addition, I reported the first synthetic gene for recombinant expression of human C5a. My lab was the first to clone human and mouse C5a receptors. My group also reported the first knockouts for C5a, C3a and C5L2. See, for example:

   - Gerard NP, GERARD C. The chemotactic receptor for human C5a anaphylatoxin. *Nature* 1991; 349: 614-617.
   - GERARD C, Bao L, Orozco O, Pearson M, Kunz D, Gerard NP. Structural diversity in the extracellular faces of peptidergic G-protein-coupled receptors. Molecular cloning of the mouse C5a anaphylatoxin receptor. *J Immunol* 1992; 149: 2600-2606.
   - Hopken UE, Lu B, Gerard NP, GERARD C. The C5a chemo attractant receptor mediates mucosal defense to infection. *Nature* 1996; 383: 86-89.
   - Hopken UE, Lu B, Gerard NP, GERARD C. Impaired inflammatory responses in the reverse arthus reaction through genetic deletion of the C5a receptor. *J Exp Med* 1997; 186: 749-756.
   - Humbles AA, Lu B, Nilsson CA, Lilly C, Israel E, Fujiwara Y, Gerard NP, GERARD C. A role for the C3a anaphylatoxin receptor in the effector phase of asthma. Nature 2000; 406: 998-1001.
   - Okinaga S, Slattery D, Humbles A, Zsengeller Z, Morteau O, Kincade MB, Brodbeck RM, Krause JE, Choe H, Gerard NP, GERARD C. C5L2, a nonsignaling C5A binding protein. *Biochem.* 2003 Aug 12; 42(31):9406-15.

6. I have reviewed the Office Action and understand that the Patent Office has taken the position that the Invention would be obvious to a person skilled in the art in light of Sato (1999), Roebroek *et al.* (2003), Homanics (2002), Lester *et al.* (2003), Champtiaux and Changeux (2002), Girardi *et al.* (2003) and Burmer *et al.* (WO 02/61087).

7. Sato and Roebroek *et al.* are cited as disclosing techniques for knocking-out an endogenous gene in a mouse, and Homanics, Lester *et al.* and Champtiaux and Changeux are cited as disclosing the disruption of mouse receptor genes and the replacement of the genes with homologous human receptor cDNA. Girardi *et al.* are cited as disclosing a

mouse with the C5aR gene knocked-out, and Burmer *et al.* are cited as disclosing human C5aR cDNA.

8. It is my understanding that the Patent Application and the cited publications are to be viewed from the perspective of one of ordinary skill in the art in the relevant field (a "Skilled Person") at the time of filing of the Patent Application in question. I have been asked to consider this time to be the period around or before 24 December 2003 (the "Relevant Period"). I would expect a Skilled Person in the field of antibody therapy during the Relevant Period to have been represented by a scientist with a Ph.D. degree in Biochemistry and/or at least 3 to 5 years experience in the field of Biochemistry, or an educational background at the same degree level in a related field and equivalent level of experience.

9. I am very familiar with the technical field of the claimed invention. I am qualified to analyze literature in this field and to provide my opinion as to what literature in this field discloses or suggests to the Skilled Person at the Relevant Period.

10. By the Relevant Period I had attained at least the level of such a Skilled Person, and further in view of my qualifications discussed above, I believe that I am qualified by training and experience to address what a Skilled Person would have understood from reading the Patent Application and the cited publications.

11. None of the documents referred to in paragraphs 6 and 7 above suggest to me now or would have suggested to me in December 2003 to introduce a humanised C5aR gene into a rodent. These documents are at best a collection of papers describing techniques for generating knock-in or knock out mice, with a paper describing the human C5aR sequence thrown into the mix.

12. Further, if it had been suggested to me around December 2003 that human C5aR signalling would be activated by mouse C5a and would function in an *in vivo* mouse model I would have been skeptical based on my knowledge at the time of C5aR and related receptors. Success in making such a mouse transgenic for human C5aR and having signaling through the human C5aR activated by mouse C5a could not have been reasonably predicted around December 2003.

13. C5aR is a G protein coupled receptor that for which the ligand (C5a) is a large peptide. G protein coupled receptors with large peptide ligands typically have as little as 70% amino acid identity between species. Most of the diversity in these G protein coupled receptors occurs on the cell surface loops where ligand binding occurs. Thus mouse and human C5a are only ~70 % identical, and the receptors are similarly divergent.

14. As evidence of the sequence divergence, an alignment of mouse and human C5a is provided in Exhibit 2, and an alignment of the human and rodent C5aR extracellular domains involved in ligand binding are provided in Exhibit 3. This sequence diversity between human and mouse C5a and human and mouse C5aR was known by December 2003, and for this reason a Skilled Person would not in my view have predicted at that time that mouse C5a would bind human C5aR.

15. A report by Woodruff *et al.* in 2001 (Woodruff *et al.*, (2001) *Inflammation,* 25:171-177, Exhibit 4) points out difficulties in crossing species using small peptide agonists and antagonists, providing further evidence that it was unpredictable whether murine C5a would activate the human receptor when expressed *in vivo* in knock-in mice.

16. In fact, cross species functions of anaphylatoxin ligands, such as C5a, and their respective G protein coupled receptors, were well known to be unpredictable. For example, in addition to C3a and C5a, human C4a was well known as an anaphylatoxin. This was because human C4a was able to cause contractions of guinea pig ileum, and cross desensitized in this assay with C3a (Hugli *et al.* (1983) *Mol Immunol*, 20:637-645, Exhibit 5). However, when the human C3a receptor was cloned, it was demonstrated that human C4a interacted uniquely as an agonist with the guinea pig C3a receptor but did not interact at all with the human C3a receptor (Lienenklaus *et al.* (1998) *J Immunol*, 161:2089-2093, Exhibit 6).

17. On page 10 of the Office Action the Examiner refers to Cain (Biochemical Pharm., 2001, Vol 61, No 12, pg 1571-1579) to support his position that it is not unexpected that mouse C5a would bind to and effects signaling of human C5aR. The Examiner asserts that Cain "taught human, mouse and rat C5aR shared significant homology (pg 1574, Fig. 1) and

discuss two peptides that inhibit C5a binding and function at human and rat C5aRs (pg 1572, col. 1, lines 10-13)".

18. In fact, however, Figure 1 of Cain is intended to highlight the sequence *variation* between the C5aR sequences of various species, not "significant homology" as the Examiner suggests. The Cain paper begins with the observation that a known C5aR antagonist (F-[OPchaWR]) has approximately 1000-fold less affinity for C5aR on mouse cells than it does for C5aR on human cells (see abstract, first sentence). The authors hypothesize that a possible cause of this difference is the species-species *variation* in the sequence of the activation domains of C5aR (see first paragraph of the Discussion, page 1575).

19. Cain therefore provides strong evidence to support my comments in paragraph 14 above. It provides a clear example of a ligand that has high affinity for human C5aR but which exhibits wide variations in binding affinity to C5aRs from other species. The fact that the ligand showed 1000-fold less affinity in binding to mouse C5aR supports my point that it was simply not expected that a ligand that binds to mouse C5aR (e.g. Mouse C5a) would bind to and effect signaling of human C5aR.

20. On page 11 of the Office Action the Examiner states: "In addition, knockin mice having a humanized receptor were known in the art to bind the mouse ligand as exemplified by Drago (Cellular and molecular life sciences, July 2003, Vol 60, pg 1267-1280), Gu (Developmental Cell, July 2003, Vol 5, pg 45-57), Belmont (WO 2002/059263) and Kane (WO 2003/027252).

21. I have read the Drago paper and cannot see any evidence of a humanized receptor binding to a mouse ligand. The paper looks almost exclusively at the phenotype of knock-out mice in which specific subunits of neuronal nicotinic receptors have been deleted.

22. I have read the Gu paper and note that it relates to a study of the Neuropilin-1 (Npn-1) receptor which binds multiple ligands from structurally distinct families such as semaphorins (Sema) and vascular endothelial growth factors (VEGF). There is no

mention at all of comparison of ligand binding across different species, and no evidence of a humanized Npn-1 receptor binding to a mouse ligand.

23. The Kane and Belmont publications relate to transgenic animals comprising humanized calcitonin gene-related peptide (CGRP) receptor and T-cell receptor genes respectively. These receptors are not related to G protein coupled receptors and are not predictive of whether a humanized G protein coupled receptor such as C5aR would function in a mouse. My comments in paragraphs 13 and 14 above highlight peculiarities of G protein coupled receptors, such as the size of the molecules and the low sequence identity levels between different species. These peculiarities mean that the functioning of other unrelated receptors, such as those described in Kane and Belmont, are simply not predictive of the functioning of C5aR.

24. I have been involved with the biotechnology and pharmaceutical industries for over twenty years and there have been enormous resources devoted to identifying a C5aR antagonist because of the wonderful preclinical data showing this as a very attractive target in a number of diseases including sepsis, macular degeneration, asthma, arthritis, cancer, and neurodegenerative diseases. This is exactly the reason why the scientific paper describing the Invention claimed in this Patent Application was published in Nature biotechnology in 2006 (Lee *et al.* (2006) *Nat Biotechnol,* 24:1279-1284, Exhibit 7).

25. As a reviewer for the Lee *et al.* (2006) manuscript, I specifically noted several important features. One, it is extremely difficult to make neutralizing monoclonal antibodies to G protein coupled receptors, and a transgenic rodent expressing functional human C5aR provides a novel and unique opportunity to accomplish this. Second, the success of the monoclonal antibodies produced in the mouse model of arthritis and described in the manuscript provided clear proof of principle to directly advance the humanized products to man. Third, and of most relevance to the Patent Application, it provided direct evidence that murine C5a generated *in vivo* in the K/BxN model of arthritis functioned at the human C5a receptor, which to me was unexpected.

26. In my opinion, therefore, there is nothing in the cited prior art to suggest that a Skilled Person could have predicted that mouse C5a would bind to human C5aR. Furthermore, I

believe that a Skilled Person would not have predicted that human C5aR would signal *in vivo* in a mouse. I further believe that a Skilled Person would not have predicted success in making a mouse that is transgenic for human C5aR and in which C5a endogenous to the mouse binds to and effects signalling of the human or humanized C5aR. There was no reasonable predictability of success, and thus no motivation, therefore, around December 2003 for a Skilled Person to generate transgenic rodents with humanized C5aR.

27. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title XVIII of the United States Code, and that such willful false statements may jeopardize the validity of the Patent Application or any patent issuing thereon.

6/25/2010

Date

Name: Craig Gerard MD PhD

Exhibit 1

CURRICULUM VITAE

Updated 9/09

Name: Craig J. Gerard

Office Address: Perlmutter Laboratory
Enders Research Building, EN144
Children's Hospital Boston
300 Longwood Ave.
Boston, MA 02115
Phone: (617) 355-6174
email: Craig.Gerard@childrens.harvard.edu
fax: (617) 730-0240

Home Address: 376 Newton St.
Chestnut Hill, MA 024676

Date of Birth: October 16, 1954
Place of Birth: Jersey City, NJ

EDUCATION:

1976	B.A., University of Connecticut
1981	M.S., Ph.D., University of California, San Diego (Chemistry)
1985	M.D., Bowman Gray School of Medicine, Wake Forest University, Winston Salem, NC

POSTDOCTORAL TRAINING:

Internship and Residencies:

1985-1986	Intern in Medicine, Beth Israel Hospital, Harvard Medical School, Boston, MA
1986-1988	Research Resident in Medicine, Beth Israel Hospital, Harvard Medical School, Boston, MA

Fellowships:

1977-1980	Predoctoral Fellow Department of Chemistry, University of California, San Diego, CA
1981-1981	Postdoctoral Fellow, Research Institute of Scripps Clinic, LaJolla, CA
1985-1987	Research Fellow in Medicine, Harvard Medical School, Boston, MA

LICENSURE AND CERTIFICATION:

1986	Massachusetts License
1988	Certified, American Board of Internal Medicine

ACADEMIC APPOINTMENTS:

1983-1985	Instructor, Department of Biochemistry, Bowman Gray School of Medicine, Winston-Salem, NC
1985-1987	Research Fellow in Medicine, Harvard Medical School, Boston, MA
1987-1989	Instructor in Pediatrics, Harvard Medical School, Boston, MA
1989-1993	Assistant Professor of Pediatrics, Harvard Medical School, Boston, MA
1993-2002	Associate Professor of Pediatrics, Harvard Medical School, Boston, MA
2002-present	Ina Sue Perlmutter Professor of Pediatrics, Harvard Medical School, Boston, MA
2003-present	Chief, Division of Respiratory Diseases, Children's Hospital Boston, MA

HOSPITAL AND HEALTH CARE ORGANIZATION SERVICE RESPONSIBILITIES:

7/85-6/1988	Research Resident in Medicine, Beth Israel Hospital and Children's Hospital Medical Center, Boston, MA
7/88-present	Assistant in Medicine, Children's Hospital, Boston, MA
6/89-present	Staff Physician, Beth Israel Hospital, Boston, MA
6/90-present	Associate Physician, Beth Israel Hospital and Beth Israel Deaconess Medical Center, Boston, MA
9/91- present	Staff Physician, Brigham and Women's Hospital, Boston, MA
6/93 - present	Associate in Medicine, Children's Hospital, Boston, MA
6/96 – 8/07	Director, Adult Cystic Fibrosis Program, Children's Hospital,Boston,MA
8/03- present	Chief, Division of Respiratory Diseases, Children's Hospital, Boston, MA

MAJOR ADMINISTRATIVE RESPONSIBILITIES:

1994	Director, Ina Sue Perlmutter Laboratory, Children's Hospital, Boston, MA
2000	Director of Research, Division of Respiratory Diseases, Children's Hospital, Boston, MA
2003	Chief, Division of Respiratory Diseases, Children's Hospital Boston, MA

MAJOR COMMITTEE ASSIGNMENTS:

1989-1993	PhD Thesis Committee member, Lois Wetmore, DVM, Department Physiology, Harvard School of Public Health
1994	PhD Thesis Committee member, Peter Barrett, Department of Genetics, Harvard Medical School
1997	Pediatric AIDS Think Tank
1997	NIH AIDS Vaccine Research Committee Focus Group
1997	PhD Thesis Committee member, Michael Farzan, Section of Virology, Harvard Medical School
1999	PhD Thesis Committee member, Carlo Rizzuto, Section of Virology, Harvard Medical School

2000	Permanent Member, Lung Biology and Pathology Study Section, NIH
2000	Permanent Member, Immunology Merit Review Section, Veterans Administration

PROFESSIONAL SOCIETIES:

1984-	American Society of Biochemistry and Molecular Biology
1991-	Cystic Fibrosis Genetic Analysis Consortium
1992-	American Society for Clinical Investigation
1995-	Society for Pediatric Research

EDITORIAL BOARDS:

1992	Reviewing Editor, Journal of Biological Chemistry
1992-	Reviewing Editor, Journal of Immunology
1994-	Reviewing Editor, Science
1994-	Reviewing Editor, Nature
1995-2005	Editorial Board, Journal of Biological Chemistry
2000-	Reviewing Editor, Nature Immunology

AWARDS AND HONORS:

1978-1981	NIH Predoctoral Fellow, UCSD, LaJolla, CA
1984	Alpha Omega Alpha
1989-1992	RJR Nabisco Faculty Scholar, Harvard Medical School
1992	American Society for Clinical Investigation

NARRATIVE REPORT:

Major Research Interests:
G-protein coupled receptors in host defense, Molecular Biology and Genetics of Inflammation, Chemokines and Viral Pathogenesis

Description of Research:

Research in the Gerard Laboratory explores the molecular mechanisms by which leukocytes traffic in host defense and inflammatory responses. Particular focus is established on the family of G protein coupled receptors, which serve to direct migration of leukocytes in both the basal and inflamed state. These include receptors for C5a and C3a anaphylatoxins, tachykinins (substance P and related molecules), lipids including platelet activating factor and leukotrienes, bacterial formyl peptides and chemokines. Both mouse genetic models and in vitro transfection and expression analyses are used to understand the complex balance between defense and untoward inflammation mediated by these mediators and receptors. While the research is basic, implications for the development of therapeutics involves many disease states, including cystic fibrosis and asthma (untoward host defense/inflammatory responses) and acquired immunodeficiency syndrome (AIDS). The chemokine family of receptors plays a special role as co-receptors for human and simian immunodeficiency viruses. The mechanisms, by which these receptors contribute to disease pathogenesis, and implications for vaccine

development, are priorities in the laboratory. Additionally, chemokines and their receptors play additional roles in viral pathogenesis for other pathogens, including herpes viruses, which have incorporated viral chemokine and chemokine receptor homologues for presently unclear reasons.

RESEARCH FUNDING INFORMATION:

1988-1993	NIH R01 HL41277, PI, 'Structure and Regulation of Human Lung Mucus Glycoprotein'
1989-1995	RJR Nabisco, PI, Faculty Scholar's Award
1990-1995	Pfizer, PI, Characterization of G-Protein Coupled Receptors
1991-1996	NIH SCOR HL19170 Director, Project 3 'Molecular Biology of Human Neutral Endopeptidase'
1994-1999	NIH RO1 HL52503, PI, 'Biology and Biochemistry of Endopeptidase 3.4.24.11'
1994-2007	NIH RO1 HL51366, PI, 'Molecular Pathology of Chronic Lung Disease'
1994-2010	NIH RO1 HL36162, Collaborator, 'Immunopharmacology of Complement Anaphylatoxins in Lung'
1994-1997	LeukoSite, PI, 'Investigation of a Putative Rantes Receptor on Eosinophils'
1996-2007	NIH RO1 AI/HL39759, PI, 'Asthma, Airway Inflammation and Beta Chemokine Receptors'
1997-2002	NIH R01 AI141851, Co-PI, 'Molecular Interaction Between HIV-1 gp120-CD4 Complexes and Chemokine Receptors'
1999-2004	NIH R01HL63645, PI, 'Biochemistry of CCR5/ CXCR4/ GP120 Signal Transduction'
2001-2010	NIH R01HL69511, PI, 'Mediators and Mechanisms of Innate Immunity in the Lung'

MAJOR TEACHING CONTRIBUTIONS:

1. Local Contributions:

a. Medical and Graduate School Courses

1992–1996	Markey Scholars Biochemistry Course, Harvard Medical School, Lecturer 20--25 Graduate Students, 20 hrs. preparation and contact time
1992–1996	Revisiting Biomedical Sciences and Pathology, Harvard Medical School, Lecturer, 25 Graduate Students, 20 hrs. preparation and contact time

b. Seminars, Invited Teaching Presentations

1993	"Seven Transmembrane Domain Receptors and Inflammation" Children's Hospital, Boston, MA
1994	"Chemoattractants, Chemokines and Granulocyte Trafficking" Massachusetts General Hospital, Boston, MA
1995	"Cystic Fibrosis" Cambridge Hospital, Boston, MA

1995 "Cystic Fibrosis" Children's Hospital, Boston, MA
1995 "Chemoattractants, Chemokines and White Cell Trafficking" Pulmonary/Critical Care Conference, Beth Israel Hospital, Boston, MA
1996 "Chemokines: from Chemotaxis to HIV" Department of Immunology, Boston University, Boston, MA
1996 "Chemoattractant Receptors in Host Defense and Inflammation" Children's Hospital Research Seminar, Boston, MA
2000 "Revisiting Biochemical Sciences and Pathology " Harvard Medical School, Boston, MA
2000 "Cystic Fibrosis: Progress in the Last Decade" Harvard Medical School, Department of Pathology, Boston, MA
2000 "Chemokine Receptors in Asthma And Transplant Rejection" Children's Hospital, Boston, MA
2001 "Clinical and Molecular Aspects of CF" Harvard Medical School, Boston, MA

c. Advisory and Supervisory Responsibility in Clinical Setting

1998 Attending Physician, Janeway Medical Service, Children's Hospital, 300 hr/yr
1988 Supervisor for 15 medical students and house officers, inpatient and outpatient service
1991 Ph.D. Thesis adviser, 2 graduate students 500-600 hr/yr
1988 Mentor, 6-9 research fellows, post-graduate MD's or PhD's, 1000 hr/yr
2003 Ph.D. Thesis adviser, 2 graduate students 500-600 hr/yr

d. Leadership Roles

1998 Grand Rounds, University of Vermont School of Medicine, Burlington, VT
1999 Grand Rounds, Lahey Clinic, Burlington, MA
2000 Cystic Fibrosis Rounds, The Children's Hospital

e. Trainees

1985-1987	Elizabeth Tam, MD	Chair of Medicine, University of Hawaii School of Medicine
1987-1989	Mark Hodges, MD	Pulmonary Physician, Baton Rouge General Medical Center, LA
1990-1992	Dieter Kunz, PhD	Faculty Molecular Biology, Basel Institute for Immunology
1990-present	Bao Lu, MD	Assistant Professor of Pediatrics, Harvard Medical School
1991-1992	Jean-Luc Paquet, PhD	Research Scientist in Neuroscience, Marion Merrill Dow, Strasburg, France
1991-1993	Levi Garraway, MD, PhD	Assistant Professor and Medical Oncologist, Dana Farber Cancer Research Institute, Broad Institute of Harvard and MIT, Boston, MA

1992-1998	Carmen Bozic, MD, CM	Vice-president Drug Safety and Risk Management, Biogen
1992-1994	Xiao-Ping He, MD	Research Associate, Department of Biochemistry, St. Jude's Hospital, Memphis, TN
1993-1995	Wendy Wai, MD	Private Practice, Dermatology
1993-1996	Lois Wetmore, DVM, DSc	Chief, Large Animal Anesthesia, Tufts University Veterinary School, Southboro, MA
1994-1995	Claudia von Uexkull, PhD	Out of Science
1994-1998	Peter Barrett, PhD	Post Doctoral Fellow, Developmental Immunology, Mass General Hospital
1994-1996	Theodore Post, MD	Nephrology Practice, Chestnut Hill, MA
1994-1995	Lee F. Kolakowski, PhD	President and CEO, ReceptorBase, Inc. Timonium, MD
1994-1997	Uta Hoepken, PhD	Instructor, Max-Delbrueck, Center for Molecular Biology, Berlin, FRG
1994-1995	M. Carmen Rodriquez-Garcia, PhD	Hospital Clinico Universitario, Unidade de Investigacion, Avda Ramon y Cajal 3, 47011 Valladolid, Spain
1996-1998	Kathleen Martin, PhD	Research Assistant Professor of Surgery and Pharmacology & Toxicology, Dartmouth Medical School
1996-1998	Allison Farrell, PhD	Senior Editor Research Manuscripts, Nature Medicine
1996-2005	Alison Humbles, PhD	Associate Scientist, MedImmune, Inc
1997-2004	Olivier Morteau, PhD	Associate Scientist
1997-1998	Miriam Schizer, MD	Pediatric Practice, Wellesley Hills, MA
1997-1999	David Jarmin, PhD	
1998-2001	Dubhfeasa, Slattery, MB, BEH, BAO, BA	Consultant in Pediatric Medicine, Children's Hospital, Dublin
1998-2003	Shoji Okinaga, MD	Department of Geriatric and Respiratory Medicine, Tohoku University School of Medicine, Japan
2000-2006	Zusanna Zsengeller, MD, PhD	Staff Scientist, Inotek Corp Beverly, MA
2001-2003	Jinming Gao, MD	Department of Respiratory Disease, Peking Union Medical College Hospital, China
2003-2004	Pixu Liu, PhD	Research Fellow, Harvard Medical School, Children's Hospital
2004-2006	David Nelson, PhD	Staff Scientist, Dana Farber Cancer Research Institute
2003-2006	Marwan Dib, MD, Ph.D.	Medical Resident
2004-2007	Sorina Ghiran, M.D.	Research Assistant, Beth Israel Deaconess Medical Center

2005-2007	Olga Borchakova, Ph.D.	Postdoctoral Fellow, Beth Israel Deaconess Medical Center
2006-present	Claire Bamberg, M.D., PhD	Postdoctoral Fellow, Children's Hospital, Harvard Medical School

2. Regional, National, and International Contributions:

a. Invited Presentations

1992 "Receptor Mediated Signaling in Inflammatory Cells" The Scripps Research Institute, LaJolla, CA
1993 "Seven Transmembrane receptors of the Leukocyte" Cardiovascular, Lung and Blood Diseases Frontiers Symposium, NIH, Rockville, MD
1994 "Targeted Disruption of CD/10 Neutral Endopeptidase" American Society of Hematology, Nashville, TN
1995 "C-X-C Chemokine Signal Transduction" and "C-C Chemokine Receptors" Fourth International Chemokine Symposium, University of Bath, Bath, United Kingdom
1995 "Cell Biology of the Host Response to Injury" Medical College of New Jersey, Newark, NJ
1995 "Chemoattractants, Chemokines and White Cell Trafficking" Pulmonary/Critical Care Conference, Beth Israel Hospital, Boston, MA
1995 "C-C Chemokine Receptors" International Chemokine Symposium, Brighton, UK
1995 "Eosinophil Chemoattractant Receptors" Fifth Annual Lung Cell Biology Symposium, Woods Hole, MA
1995 "Chemoattractants, Chemokines and White Cell Trafficking" W. Allan Tisdale Medical Sciences Grand Rounds, University of Vermont School of Medicine, Burlington, VT
1995 "PAF Receptor Anchors Streptococcus Pneumoniae To Activated Human Endothelial Cells" 5th International Congress on Platelet-Activating Factor and Related Lipid Medicators, Berlin, FRG
1996 "Transgenic Models in the Study of Inflammatory Disease" University of Cincinnati Medical Center, Cincinnati, OH
1996 "Signaling Pathways in Lung Inflammation" American Physiological Society Symposium, FASEB Meeting, Atlanta, GA
1996 "Chemotactic cytokines in Inflammation" National Jewish Center for Immunology and Respiratory Medicine, Denver, CO
1996 "Early Disease-How does it Start?" Cystic Fibrosis Foundation, Williamsburg Conference, Williamsburg, VA
1996 "HIV and Chemokines" NIH, Washington, DC
1996 Third Annual Conference on Chemokines, IBC, San Francisco, CA
1996 Symposium, New Drugs for Inflammatory, Allergic, and Immunologic Diseases, Hershey, PA
1996 "Chemoattractant Receptors and Host Defense" Inflammation Research Society, Woods Hole, MA

1996 "The Biology of Chemoattractant Receptors" Cardiovascular Research Institute, University of California, San Francisco, CA
1997 "Genetic and Biochemical Analysis of Chemokine Receptors" Biology and Physiology of Chemokines, Centro Nacional De Biotecnologia, Madrid, Spain
1997 "The Role of Chemokines in Leukocyte Trafficking and Disease " Keystone Conference, Copper Mountain, CO.
1997 "Gene Targeting of the Chemoattractant Receptor Genes" International Symposium on the Molecular Cell Biology of Macrophages '97, University of Tokyo, Tokyo, Japan
1998 "Immune Regulation" The Eighth Frank and Bobbie Fenner Conference in Medical Research, The Australian National University, Canberra, Australia
1998 "CCR3 and CXCR3 Gene Targeted Mice" Japanese Congress of Immunology, University of Tokyo School of Medicine, Tokyo, Japan
1998 "NEP Knockout Mouse" The 1st International Symposium of Cardiovascular Endocrinology and Metabolism, Kyoto, Japan
1998 "Gene Knockout of Chemokine Receptors CCR3, CXCR3, CCR1" Immunology and Allergy Week, Chemokine/Cytokine and Inflammation Symposium, Kobe, Japan
1999 "Gene Knockout of the Eotaxin Receptors " Chemokines and Their Receptors: From Basic Research to Therapeutic Intervention, Institut Pasteur, Paris, France
1999 Invited International Judge, International Jury for the Francqui-Prize 1999, Francqui Foundation, Brussels, Belgium
1999 "Regulation of Cellular Processes by Infectious Microbes" AFMR Experimental Biology Conference, Washington, DC
1999 "Animal Models" Cystic Fibrosis Foundation's Williamsburg Conference, Williamsburg, VA
1999 "Regulation of Inflammation II" 1999 Phagocytes Gordon Research Conference, New London, CT
1999 "Overview of Chemokine Biology" 1999 Gordon Research Conference on Medicinal Chemistry, New London, NH
2000 "Chemokine Receptor knockouts: What Have We Learned?" 2000 AIC Conference on Immunology, Chicago, IL
2000 "G-Protein Coupled Receptors in Lung Inflammation" Thomas Petty Conference, Aspen, CO
2000 "GPCR's: from asthma to AIDS" Foundation Franqui, Brussels, France
2000 "Modulation of Infectious Disease in Chemokine Receptor Knockout Mice" Keystone Symposia 2001, Taos, New Mexico
2000 "11th International Congress of Immunology", Invited Session Chairperson Stockholm, Sweden
2002 "Chemokine Conference 2002", Madrid, Spain
2003 "Arthritis Reseach Conference 2003", Keystone, CO
2003 "6th World Congress of Inflammation, Vancouver, Canada

CLINICAL ACTIVITIES:

1. Report of Clinical Activities:

a. Internal Medicine, adult cystic fibrosis,
Children's, Beth Israel Deaconess and Brigham and Women's Hospital

b. Time Commitments

patient care	20%
teaching	30%
administration	20%
research	30%

c. Patient load
Inpatient and outpatient care as required for 40 chronically ill patients

BIBLIOGRAPHY

Original Articles:

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Exhibit 2

Mouse C5a vs Human C5a

63.5% identical

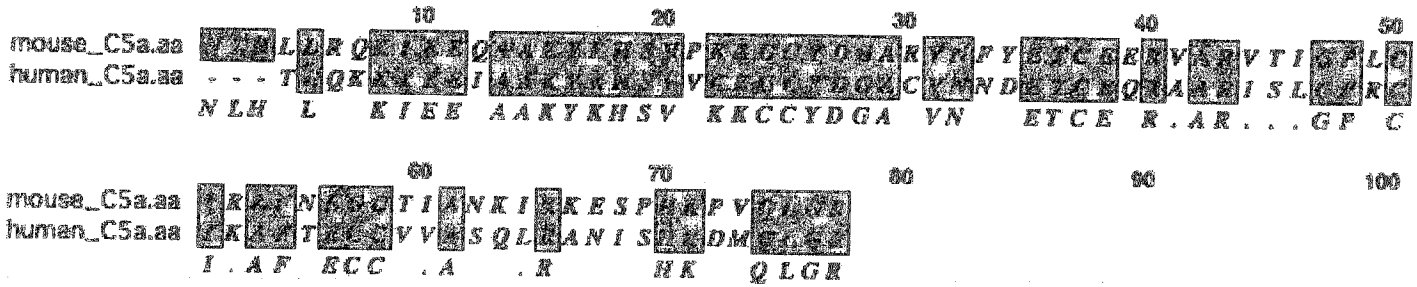

EXHIBIT 2

Exhibit 3

Alignment of C5aR extracellular domains

mouse vs. human: 29.8% identical
rat vs. human: 29.8% identical

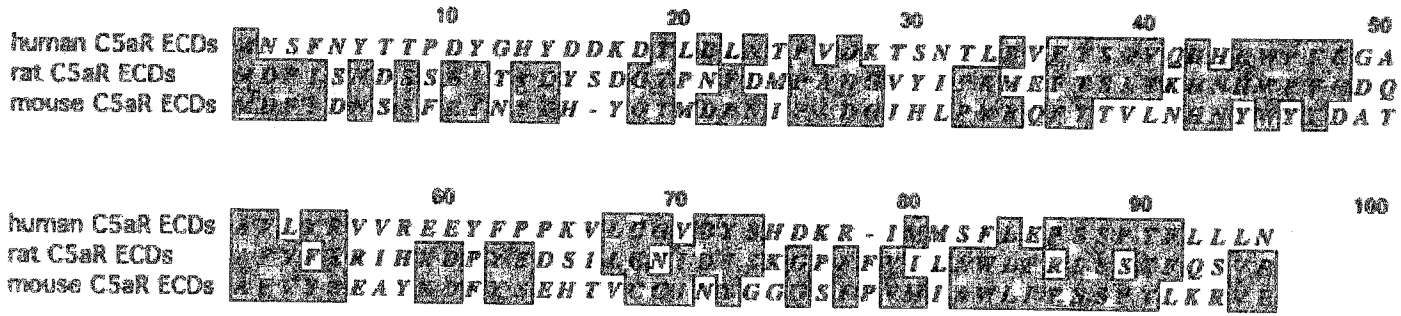

EXHIBIT 3

Exhibit 4

EXHIBIT 4

Inflammation, Vol. 25, No. 3, 2001

Species Dependence for Binding of Small Molecule Agonist and Antagonists to the C5a Receptor on Polymorphonuclear Leukocytes

Trent M. Woodruff,[1] Anna J. Strachan,[1] Sam D. Sanderson,[4] Peter N. Monk,[3] Allan K. Wong,[2] David P. Fairlie,[2] and Stephen M. Taylor[1]

Abstract—This study investigated the receptor binding affinities of a C5a agonist and cyclic antagonists for polymorphonuclear leukocytes (PMNs) isolated from human, sheep, pig, dog, rabbit, guinea pig, rat and mouse. The affinities of the two small molecule antagonists, F-[OPdChaWR] and AcF-[OPdChaWR], and the agonist, YSFKPMPLaR, revealed large differences in C5a receptor (C5aR) affinities between species. The antagonists bound to human, rat and dog PMNs with similar high affinities, but with lower affinities to PMNs from all other species. The C5a agonist also bound with varying affinities between species, but showed a different affinity profile to the antagonists. In contrast, recombinant human C5a had similar affinity for PMNs of all species investigated. The low correlation between the affinities of the antagonists and the agonist between species either suggests that different receptor residues are important for distinguishing between agonist/antagonist binding, or that the agonist and antagonist peptides bind to two distinct sites within the C5aR.

KEY WORDS: C5a antagonist; C5a agonist; leukocytes.

INTRODUCTION

The activation of the blood complement cascade results in the cleavage of the molecule C5 leading to production of the anaphylatoxin C5a. This molecule is a potent inflammatory mediator that causes chemotaxis of inflammatory cells, increased vascular permeability, spasmogenesis, immunoregulation and the release of a variety of inflammatory cytokines (1–5). A combination of these events typifies the normal inflammatory response.

C5a exerts its biological actions by binding to the plasma membrane bound C5a receptor (C5aR), a member of the G-protein coupled superfamily (6). The C5aR has been sequenced in numerous species including human, mouse, dog, rat, guinea pig, rabbit and several non-human primates (chimpanzee, gorilla, orang-utan and rhesus macaque) (7–12). Interestingly, there is only about 70% amino acid homology between the non-primate species. This low degree of similarity is unusual for members of the G-protein coupled superfamily, which typically possesses 85–98% interspecies sequence homology (13).

Due to the proinflammatory actions of C5a, excessive or inappropriate C5a activity may cause prolonged inflammation, and lead to disease pathology. Anaphylotoxin C5a has been implicated in numerous inflammatory diseases including rheumatoid arthritis, Alzheimer's disease, endotoxemia and adult respiratory distress syndrome (14–19). To date, no drug aimed at inhibiting the complement system is available clinically to control these diseases. An agent developed to block the actions of C5a is thus highly desirable.

[1] Department of Physiology and Pharmacology.
[2] Centre for Drug Design and Development, University of Queensland, Australia.
[3] Department of Molecular Biology and Biotechnology, University of Sheffield, United Kingdom.
[4] Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha.

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We have developed a series of low molecular weight antagonists for the C5aR, which bind with high affinity to isolated human PMNs and display potent inhibitory activities towards C5a-mediated myeloperoxidase release on human PMNs (20, 21). In this study, the relative binding affinities of the two cyclic antagonists, Phe[L-ornithine-Pro-D-cyclohexylalanine-Trp-Arg] (F[OPdChaWR]) and AcPhe[L-ornithine-Pro-D-cyclohexylalanine-Trp-Arg] (AcF-[OPdChaWR]), were examined in various species of PMNs (human, sheep, pig, dog, rabbit, guinea pig, rat and mouse), in order to compare with the affinities seen in human PMNs. Additionally, the binding affinities of the C5a agonist, Tyr-Ser-Phe-Lys-Met-Pro-Leu-D-Ala-Arg (YSFKPMPLaR) (22), and recombinant human C5a (rhC5a) were determined in PMNs of the above mentioned species.

MATERIALS AND METHODS

Synthesis of C5a Antagonists and Agonist Peptides. Antagonist peptides were synthesised as described previously (18, 20, 23). Briefly, the linear peptides FOPdChaWR and AcFOPdChaWR were synthesised initially using in situ neutralization methods on a butoxycarbonyl (Boc)-Arg(Tosyl)-Pam resin. These linear peptides were then cyclised using 5 eq benzotriazole1-yl-oxy-tris-(dimethylamino)-phosphoniumhexa-fluorophosphate and 10 eq diisopropylethylamine in dimethyl-formamide (10^{-4} M, 15 h), followed by purification by HPLC. The C5a agonist, YSFKPMPLaR, was also synthesised as described previously (22, 24).

Cell Isolation. Whole blood (10–25 mL) was collected from animals via venipuncture (human, sheep, pig, rabbit and dog) or cardiac puncture (guinea pig, rat and mouse; under anaesthesia), and placed in a tube containing heparin (2 units/mL blood). The blood was then layered over an equal volume of Histopaque 1077 (Sigma, U.S.A.) and centrifuged at 400 × g for 30 min at room temperature (25°C). The top layer of platelet-rich plasma, the monocyte and lymphocyte interface, and the separation layer of histopaque were removed and discarded, leaving the PMN and red blood cell-rich layer. Red blood cells were lysed by adding distilled water (4°C) and mixing for 40 s. Isotonicity was restored to the cells through the addition of concentrated (10×) Dulbecco's phosphate buffered saline (Sigma, U.S.A.) at a volume 1/10th that of the distilled water added. The cells were then centrifuged at 700× g for 20 min at 4°C. The supernatant of lysed red blood cells was removed until 5–7 mL of PMN-rich solution remained, and another lysis performed. Phosphate buffered saline (10×) was again added at 1/10th volume followed by centrifugation at 700 × g (20 min, 4°C). The supernatant was then removed leaving a pellet of PMNs which were resuspended in buffer containing 50 mM HEPES, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5% BSA, 0.1% bacitracin and 100 μM phenylmethylsulfonyl fluoride (Sigma, U.S.A.), at pH 7.4. Resuspended cells were then counted on a hemocytometer and made up to a final density of 4×10^6 cells/mL in buffer and stored on ice until use in the receptor binding assay. Cell purity was determined through staining with xanthene and thiazine dyes with a Rapid Diff Kit (Universal Diagnostics, Australia) revealing routine purity of approximately 95–99% PMNs.

Receptor Binding Assay. Assays were performed in Millipore Multiscreen 96-well filtration plates (GV 0.22 μM). The plate was washed with 100 μL of buffer to saturate the filters. Increasing concentrations of recombinant human C5a (rhC5a; Sigma, U.S.A.), C5a agonist or antagonist peptides were added, along with ^{125}I-rhC5a (20,000 counts/well, approximately 50 pM; NEN, U.S.A.) and isolated cells (2×10^5), to a final volume of 200 μL/well with buffer. The plate was then incubated for 60 min at 4°C, followed by filtration and washing with 100 μL buffer. Filters were dried and radioactivity measured on a LKB gamma counter.

Statistical Analysis. For each experiment, non-specific binding data (values in presence of 100 nM rhC5a) were subtracted from each data point to obtain specific binding. A non-linear regression analysis using Graph Pad Prism 2 software (Graph Pad software) was performed, resulting in a concentration-response curve, where IC_{50} values (concentration of peptide resulting in 50% inhibition of ^{125}I-rhC5a binding) and pD_2 ($-\log IC_{50}$) values were obtained for each peptide in each species. A log potency ratio (Δ values; [$-\log IC_{50}$ rhC5a] − [$-\log IC_{50}$ peptide]) for each peptide in each species was also calculated. The pD_2 and Δ values for each peptide from individual experiments were used to determine arithmetic means ± standard error (*SE*). Statistical significance between peptide affinities in each species was determined with a one-way analysis of variance, followed by a Newman-Keuls post-hoc test, performed on both pD_2 and Δ values using Graph Pad Prism 2 software. Differences were considered significant when $P < 0.05$.

RESULTS

The C5aR binding affinities for rhC5a in PMNs of the species examined (human, sheep, pig, dog, rabbit, guinea pig, rat and mouse) were found to be similar (Figure 1, Table 1). As compared to C5a however, large variances in the affinities of the two C5a antagonists F-[OPdChaWR] and AcF-[OPdChaWR] were found between the various species of PMNs (Figure 1, Table 2). Of the species examined, only dog and rat displayed similar affinities to the human PMN. The remaining species (sheep, pig, rabbit, guinea pig and mouse) showed significant ($P < 0.01$) decreases in C5aR affinities of both antagonists compared to human.

In the next set of experiments, the relative binding affinities for the small molecule C5a agonist, YSFKPMPLaR, were examined. These results, shown in Figure 1 and Table 3, indicate that like the small molecule antagonists, there was a large variation in binding affinities between the species examined. Unlike the antagonists however, it was only dog PMNs that displayed similar binding affinities to human PMNs. All other

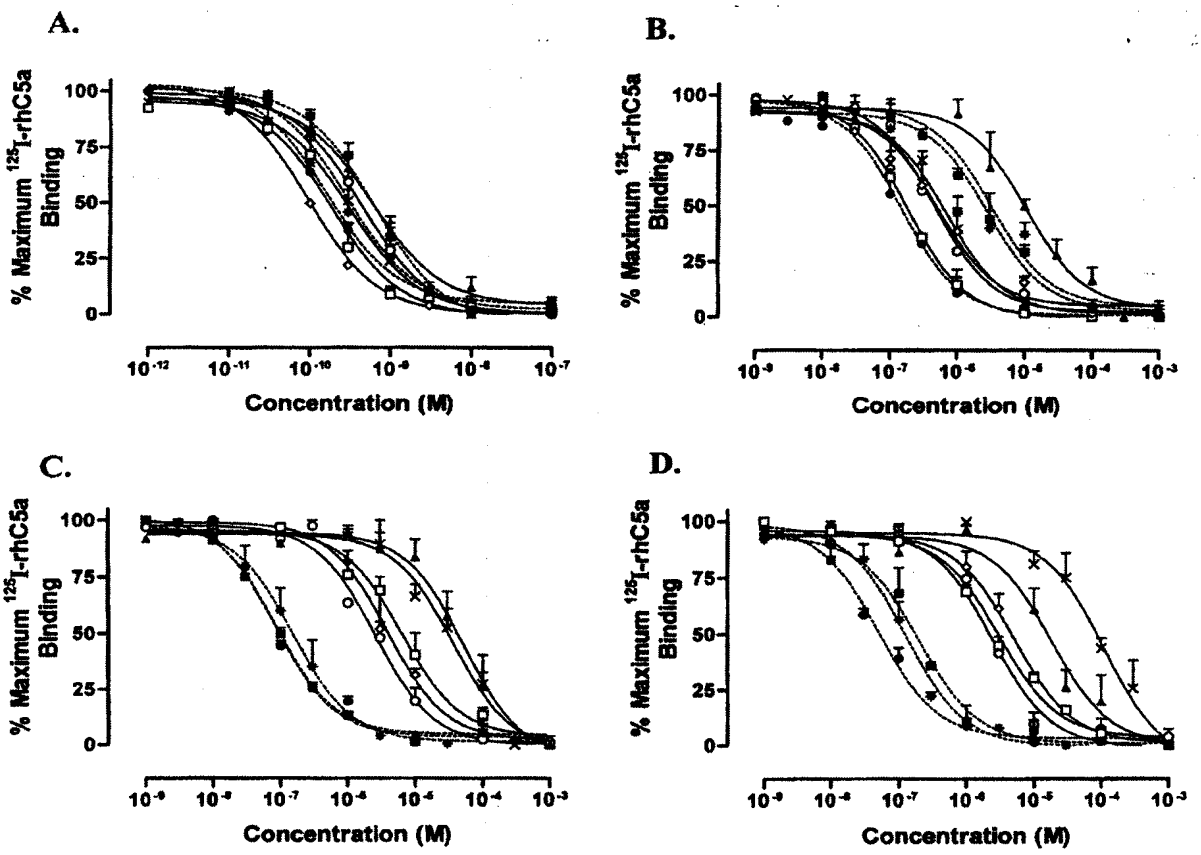

Fig. 1. Inhibition of ^{125}I-rhC5a Binding to Isolated PMNs of Various Species by C5a, C5a Antagonists and a C5a Agonist. Shown are the receptor binding curves determined for A. recombinant human C5a B. YSFKPMPLaR C. AcF-[OPdChaWR] and D. F-[OPdChaWR] in human (■), dog (⋆), rat (●), sheep (◇), rabbit (□), guinea pig (○), pig (▲) and mouse (×). Dotted line shown for human, dog and rat. Isolated PMNs from each species were incubated at 4°C with antagonist and ^{125}I-rhC5a for 60 min, then filtered and radioactivity counted. Each point represents the mean value with error bars denoting *SE* ($N = 3$–8).

Table 1. Receptor Binding Affinities of rhC5a in PMNs from Various Species

Species	n	$pD_2 \pm SE$	IC_{50} (nM)
Human	8	9.21 ± 0.07	0.61
Dog	4	9.49 ± 0.16	0.33
Rad	5	9.52 ± 0.29	0.30
Sheep	3	10.05 ± 0.02	0.09
Rabbit	3	9.65 ± 0.03	0.23
Guinea Pig	3	9.36 ± 0.02	0.44
Pig	4	9.22 ± 0.09	0.60
Mouse	6	9.53 ± 0.23	0.30

species, apart from pig, displayed a significant ($P < 0.05$) increase in affinity towards YSFKPMPLaR compared to human, with pig showing a decrease in affinity.

This variation in the binding behavior between the two antagonists, AcF-[OPdChaWR] and F-[OPdChaWR], and the C5a agonist, YSFKPMPLaR, led to an examination of the correlation of these compounds' affinities in various species between each other. The two antagonists displayed strong correlation ($r^2 = 0.937$; $P < 0.01$), while the C5a agonist YSFKPMPLaR had poor correlations between either the non-acetylated ($r^2 = 0.05$; $P > 0.05$) or acetylated ($r^2 = 0.05$; $P > 0.05$) antagonist (Figure 2).

DISCUSSION

The C5aR affinities for the two antagonists, F-[OPdChaWR] and AcF-[OPdChaWR], have already been determined in isolated PMNs of humans and rats, with both agents showing similar affinities relative to one another as well as in both species of PMNs (18, 20, 25). Prior to the present study however, no other species of PMNs had been examined. In this study, the additional species; dog, sheep, rabbit, guinea pig, pig and mouse, were chosen for C5aR affinity determination to compare with human PMNs. A competition binding assay was employed to determine receptor affinities where each peptide tested competed with ^{125}I-rhC5a for the C5aR in isolated PMNs of each species. This assay would prove to be effective only if rhC5a did in fact bind to C5aRs in different species, since the low sequence homology of C5aRs between species could markedly affect the binding of rhC5a. Previous studies however have shown that rhC5a can bind to C5aRs from the mouse, dog, rat, guinea pig and rabbit (7, 8, 10–12), indicating that rhC5a may be used experimentally in each species. In the present study, rhC5a was found to bind effectively, and with reasonably similar affinity to PMNs of all species examined (Figure 1). In fact, rhC5a was found to bind

Table 2. Receptor Binding Affinities of C5a Antagonists in PMNs from Various Species

		F-[OPdChaWR]		
Species	n	$pD_2 \pm SE$	IC_{50} (μM)	Δ Value[a] ± *SE*
Human	3	6.67 ± 0.18	0.21	2.57 ± 0.23
Dog	4	6.90 ± 0.10	0.13	2.59 ± 0.10
Rat	3	7.31 ± 0.05	0.05	2.47 ± 0.14
Sheep	3	5.28 ± 0.08[b]	5.21	4.78 ± 0.10[b]
Rabbit	3	5.42 ± 0.06[b]	3.76	4.22 ± 0.06[b]
Guinea Pig	3	5.75 ± 0.04[b]	1.79	3.62 ± 0.04[b]
Pig	4	4.53 ± 0.26[b]	29.4	4.69 ± 0.22[b]
Mouse	4	3.73 ± 0.06[b]	187	5.73 ± 0.29[b]
		AcF-[OPdChaWR]		
Species	n	$pD_2 \pm SE$	IC_{50} (μM)	Δ Value[a] ± *SE*
Human	3	7.06 ± 0.08	0.09	2.32 ± 0.06
Dog	4	6.80 ± 0.15	0.16	2.68 ± 0.08
Rat	3	7.36 ± 0.33	0.04	2.42 ± 0.24
Sheep	3	5.31 ± 0.07[b]	4.92	4.74 ± 0.09[b]
Rabbit	3	5.17 ± 0.24[b]	6.76	4.42 ± 0.24[b]
Guinea Pig	3	5.69 ± 0.20[b]	2.04	3.67 ± 0.20[b]
Pig	4	4.35 ± 0.12[b]	44.2	4.87 ± 0.14[b]
Mouse	3	4.44 ± 0.18[b]	36.3	4.98 ± 0.24[b]

[a] Δ Value = log potency ratio; [pD_2 rhC5a]—[pD_2 peptide] in each species.
[b] Significant difference from human ($P < 0.01$, one-way ANOVA).

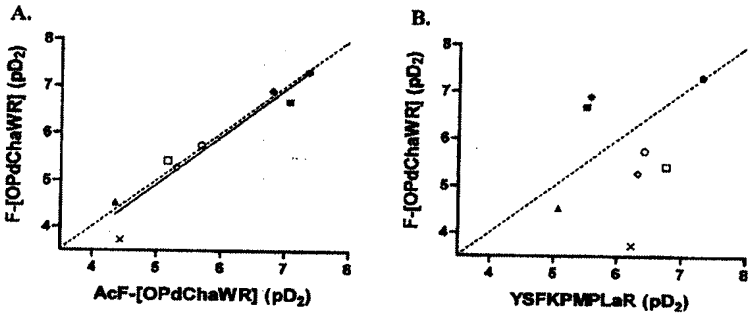

Fig. 2. Correlation of Receptor Binding Affinities of C5a Agonist and Antagonists in PMNs from Various Species. Shown are the graphs comparing the affinities between **A.** F-[OPdChaWR] and AcF-[OPdChaWR] and **B.** F-[OPdChaWR] and YSFKPMPLaR in PMNs from human (■), dog (⋆), rat (●), sheep (○), rabbit (□), guinea pig (○), pig (▲) and mouse (×). Each point represents the mean pD_2 value for each compound in each species. The dotted line in each graph represents the line of identity (pD_2 of peptide$_1$ = pD_2 of peptide$_2$). Linear regression analysis was performed for each correlation giving r^2 values of 0.93 (A; line shown; $P < 0.01$); 0.05 (B; line not shown) and 0.05 (AcF-[OPdChaWR] and YSFKPMPLaR; $P > 0.05$; graph not shown).

with a higher affinity to all other species of C5aRs compared to the human PMN C5aR (Table 1).

The next step of this study was the determination of C5aR affinities of the antagonist F-[OPdChaWR] and its acetylated counterpart, AcF-[OPdChaWR] in each of these species. There were clearly major differences in the affinity of these compounds for C5aRs from different species, with both antagonists showing similar changes across the species. There was no significant difference from human to dog to rat affinities, suggesting the potential use of these species for in vivo testing of these antagonists. For all other species tested (sheep, rabbit, guinea pig, pig and mouse), a large increase in the estimated in vivo dosage of the antagonists would be necessary to overcome the decreased in vitro receptor affinities found in these species.

A likely reason for the large change in affinities of the C5a antagonists between species is due to variances in the C5aR receptor sequences. These small antagonists are likely to be more sensitive to subtle differences in receptor sequences than the larger C5a molecule, which binds to a larger surface area of the receptor at multiple sites (27) and which shows no significant differences in affinity between species.

The C5a agonist, YSFKPMPLaR, also displayed large variations in C5aR affinities between the various

Table 3. Receptor Binding Affinities of the C5a Agonist, YSFKPMPLaR, in PMNs from Various Species

Species	n	YSFKPMPLaR $pD_2 \pm SE$	IC_{50} (μM)	Δ Value[a] ± *SE*
Human	5	5.49 ± 0.10	3.24	3.69 ± 0.13
Dog	4	5.56 ± 0.12	2.73	4.01 ± 0.15
Rat	3	7.30 ± 0.31[b]	0.05	2.55 ± 0.23[b]
Sheep	3	6.31 ± 0.31[b]	0.49	3.73 ± 0.28
Rabbit	3	6.75 ± 0.05[b]	0.18	2.89 ± 0.08
Guinea Pig	3	6.41 ± 0.09[b]	0.39	2.95 ± 0.07
Pig	3	5.07 ± 0.03	8.49	4.20 ± 0.08
Mouse	3	6.22 ± 0.23[b]	0.60	3.19 ± 0.41

[a] Δ Value = log potency ratio; [pD_2 rhC5a]—[pD_2 peptide] in each species.
[b] Significant difference from human ($P < 0.05$, one-way ANOVA).

species examined. Once again, the small molecular size of this compound would be expected to be more sensitive to alterations in amino-acid sequences in receptors of different species compared to C5a. Interestingly, the changes in affinity between species for this agonist were rather different to those seen with the C5a antagonists. This would suggest that the antagonists and agonist binding is influenced by different amino acids of the receptor and it is conceivable that they even bind to different sites within the C5aR.

The binding of C5a to the C5aR and its subsequent activation is thought to follow a "message-address" paradigm (26). Around half of the binding energy for C5a to its receptor comes from the interaction of the N-terminal extracellular portion of the receptor, with the highly charged 4-α-helical bundle of C5a (27, 28). On the other hand, the activating C-terminus of C5a is thought to interact with a different site on the receptor, possibly within the transmembrane helical domain (26). Although this site has yet to be properly defined, numerous reports have indicated the importance of the residues Arg_{206} and Glu_{199} in receptor activation (29–31). These residues are located in the upper 5th transmembrane domain and 2nd extracellular group respectively (29–31).

The agonist and antagonists used here have been developed from structure-activity relationships for small peptidic analogues of the C-terminus of C5a (24, 22, 32). It is reasonable to assume that these peptides bind at or near the activation site of the C5aR. Due to the disparity between species of C5aR affinities of the C5a agonist and antagonists reported here, it would be instructive to compare the various C5aR sequences for sequence variations that correlate with the observed changes in affinities. The locations of receptor residues that cause the large differences in affinities for the C5a agonist and antagonists will guide site-directed mutagenesis studies aimed at mapping the receptor binding site or sites for antagonists and the agonist (Cain et al., in press).

This study has characterised affinities of rhC5a, two cyclised peptide antagonists, and a C5a agonist for the C5aR on various species of PMNs. Although rhC5a bound with similar affinity in all species, the C5a antagonists and agonist displayed significant variations in affinities between species, which probably reflects their relative sensitivities to variances in C5aR sequences, but may also reflect different binding sites on the receptor.

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EXHIBIT 4

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Exhibit 5

EXHIBIT 5

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THE ACTIVE SITE OF HUMAN C4a ANAPHYLATOXIN

TONY E. HUGLI,* MARLEEN S. KAWAHARA,* CECILIA G. UNSON, RICARDO MOLINAR-RODE and BRUCE W. ERICKSON

*Department of Molecular Immunology, Research Institute of Scripps Clinic, La Jolla, CA 92037, U.S.A. and The Rockefeller University, New York, NY 10021, U.S.A.

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Abstract—The human C4 activation peptide C4a has recently been shown to be biologically active and to share common tissue receptors with human C3a anaphylatoxin. Human C3a and C4a each induce contraction and cause cross-desensitization of isolated guinea-pig ileal strips. The essential active site of C3a is comprised in the model peptide containing the five COOH-terminal residues, Leu–Gly–Leu–Ala–Arg. The anaphylatoxic activities of the corresponding C4a pentapeptide, Ala–Gly–Leu–Gln–Arg, and several other synthetic peptides related to the COOH-terminal sequence of human C4a were examined. The C4a pentapeptide induced contraction of guinea-pig ileum at 1×10^{-3} *M* and produced a wheal and flare reaction in human or guinea-pig skin when 2–5 μmole were injected intradermally. The corresponding C3a pentapeptide is 500-fold more active, since it induces contraction of guinea-pig ileum at $3–4 \times 10^{-6}$ *M* and only 4–10 nmole induce a visible skin reaction. Although the C4a pentapeptide is relatively inactive compared to the C3a pentapeptide, two analogs of these peptides, Leu–Gly–Leu–Gln–Arg and Ala–Gly–Leu–Ala–Arg, each exhibited significantly greater activity than Ala–Gly–Leu–Gln–Arg and each analog desensitized ileal smooth muscle towards contraction by either C3a or C4a. Thus it is a combination of two amino acid substitutions, the Ala for Leu-73 and Gln for Ala-76, in the COOH-terminal pentapeptide of C3a that accounts for the markedly reduced activity of C4a. The contribution of the COOH-terminal portion of C4a on its activity was further documented by examining the C4a octapeptide, Lys–Gly–Gln–Ala–Gly–Leu–Gln–Arg and a trialanyl analog, Ala–Ala–Ala–Ala–Gly–Leu–Gln–Arg. The C4a octapeptide, C4a (70–77), exhibited 5-fold greater biologic activity than the C4a pentapeptide, while the trialanyl analog was 40-fold more active. Anaphylatoxic activities of the C4a-(73–77) pentapeptide, C4a-(70–77) octapeptide, and the trialanyl octapeptide analog and their ability to specificially block the action of C3a and C4a on smooth muscle tissue support the conclusion that, as in C3a, the essential active site of C4a resides at its COOH terminus. Since C4a functions as an anaphylatoxin and significant quantities of this mediator may be generated in individuals with hereditary angioneurotic edema (HANE), the hypotheses that the kinin-like activity promoting edema in HANE patients is derived solely from component C2 and/or kininogens should be reappraised. The activities previously assigned to C4a and now confirmed by synthetic C4a analog peptides suggest that the kinin-like activity generated in HANE plasma may be derived in part from C4a.

INTRODUCTION

Several of the serum complement proteins (C3, C4, C5) were recently shown to be structurally related based on chemical properties common to their activation peptides (Gorski *et al.*, 1981; Fernandez & Hugli, 1977). Two of these activation peptides (C3a, C5a) are well-known anaphylatoxins, named for their similar and characteristic biologic actions of stimulating histamine release from the mast cells (Johnson *et al.*, 1979) and of contracting smooth muscle tissue (Cochrane & Muller-Eberhard, 1968). Based on detailed chemical and biological characterization, the activation peptide C4a was recently identified as a third anaphylatoxin (Gorski *et al.*, 1979*a*, *b*). The active fragments C3a, C4a and C5a are enzymatically released from precursor proteins C3, C4 and C5, respectively, in the course of complement activation. Human C3a and C4a are small (mol. wt 9000), cationic (p*I* = 9.0–9.5), non-glycosylated, single-chain polypeptides, whereas human C5a is a somewhat larger (mol. wt 11,000), glycosylated factor with a slightly smaller polypeptide chain (mol. wt 8200) that is less cationic (p*I* = 8.5–8.7) than either C3a or C4a. Since each of these activation peptides is generated from the amino terminus of the alpha chains of the respective precursor protein, similarities in primary structure of C3a, C4a and C5a provided strong evidence favoring a common genetic ancestry for the parent components C3, C4 and C5.

Convincing evidence that human C4a may function as an anaphylatoxin was reported only recently (Gorski *et al.*, 1979*b*). Previous reports concerning the activity of the C4 fragment were conflicting and inconclusive. Dias da Silva *et al.* (1967) claimed that the low mol. wt fragment of C4, designated C4a, did not contract smooth muscle, while Budzko &

Muller-Eberhard (1970) reported that approximately 10 μg of the C4a fragment induced contraction of isolated rat uterus, albeit inconsistently. The problem was complicated because the activity of human C4a is low relative to those of C3a and C5a in bioassays used conventionally to identify these factors. Confirmation of C4a as an active entity required a considerable effort and the quantities of C4a necessary for repeated testing were difficult to obtain.

More importantly, it was necessary to show that the C4a preparations were not contaminated with small quantities of C3a or C5a, since either factor could have seriously misled our interpretation of positive activity associated with C4a anaphylatoxin. Gorski *et al.* (1979*b*) eliminated the possibility of C5a contamination by showing that in the guinea-pig ileal assay pure human C4a is cross-tachyphylactic for human C3a but not for human C5a. The possibility of C3a contamination was dismissed since C3a was not detected by radioimmunoassay. Nevertheless, biologic activity associated with a synthetic peptide based on the C4a chemical structure provides perhaps the most convincing evidence that C4a exhibits anaphylatoxic properties.

Synthetic peptide analogs based on the COOH-terminal sequence of C3a exhibit biological activity and display a specificity similar to that of natural C3a (Hugli & Erickson, 1977). Specifically, the COOH-terminal pentapeptide common to human, rat and pig (C3a (Leu–Gly–Leu–Ala–Arg or LGLAR)* comprises the essential active site for eliciting inflammatory activities of these factors (Caporale *et al.*, 1977). Thus we examined synthetic peptides based on the COOH-terminal sequence of human C4a for evidence of functional activity corresponding to that of the natural factor. Clearly, activities expressed by synthetic peptides based strictly on C4a structure would confirm the assignment of C4a as an anaphylatoxin and would eliminate the possibility that factors other than C4a could account for the activities now attributed to this molecule.

The present results confirm our earlier characterization of C4a as an anaphylatoxin by showing that synthetic peptides based on the COOH-terminal sequence of human C4a are biologically active spasmogens. The synthetic C4a peptide mimics both the action and the specificity of the human C4a and identify the COOH-terminus as the essential active site of human C4a anaphylatoxin.

MATERIALS AND METHODS

Preparation of the anaphylatoxins

Human C3a was prepared from activated serum containing 1 *M* 6-aminohexanoic acid by the method of Hugli *et al.* (1975). Human C4a was prepared according to two separate isolation procedures. Initially, C4a was obtained by the method of Gorski *et al.* (1981) using purified human C4 as a substrate for human C1s, the activating enzyme of the classical complement pathway that selectively cleaves C4 into C4a and C4b. More recently, human C4a was isolated by the procedure of Hugli *et al.* (1982).

Synthesis of C4a peptides and analogs

The peptides were synthesized from Boc-amino acids by the solid-phase method (Erickson & Merrifield, 1976). The procedures of Caporale *et al.* (1980) were followed, except that the peptides were purified by high-pressure liquid chromatography on an analytical μBondpak C_{18} reverse-phase column (Waters Associates, Milford, MA).

Bioassays of the active factor

The ability of the natural and synthetic factors to contract smooth muscle was examined using the guinea-pig ileal assay (Cochrane & Muller-Eberhard, 1968). The influences of these factors on vascular permeability was estimated in human and guinea-pig skin. A known quantity was injected intradermally and the site of injection was monitored for a local response in man by recording the diameters of the visible wheal and flare (Wuepper *et al.*, 1972) and in guinea-pigs the diameter of the blue area on the underside of the animal's skin. Evans blue dye was introduced into the guinea-pig circulation prior to testing by intracardially injecting 0.5 ml of 1% dye in isotonic saline. Any sensation of pain, itching, or numbness

* Two conventions for notation of amino acid sequences have been used here. The standard three-letter code recommended by the IUPAC-IUB Commission on Biochemical Nomenclature was substituted in the text by the recommended one-letter notation in order to conserve space. Specifically; G, Gly; K, Lys; L, Leu; M, Met; Q, Gln; R, Arg; S, Ser; and V, Val.

following the injection was also recorded as a qualitative measure of the local reaction.

RESULTS

The partial COOH-terminal sequence of human C4a is KGQAGLQR (Moon *et al.*, 1981). Therefore, eight peptides that mimic or are structurally related to this COOH-terminal C4a sequence were prepared by solid-phase synthesis and purified by reverse-phase high-pressure liquid chromatography (Table 1). Included were the COOH-terminal pentapeptides of human C3a (LGLAR, residues 73–77) and C4a (AGLQR, residues 73–77), as well as four analogs substituted at positions 73 and 76, residues which differ between the C3a and C4a sequences. Peptide VGLAR represents the COOH-terminal pentapeptide of bovine C4a (Booth *et al.*, 1979; Smith *et al.*, 1982). The COOH-terminal octapeptide of human C4a, (KGQAGLQR, residues 70–77) and a trialanyl analog (AAAAGLQR) were also synthesized and characterized. Repeated chromatography of these synthetic peptides on the octadecylsilica column gave single symmetrical peaks. In addition, each peptide migrated as a single spot in three separate thin-layer chromatographic systems and gave acceptable amino acid compositions after acid hydrolysis (Table 1). These results demonstrate that the synthetic peptides are homogeneous by these analytical methods. The COOH-terminal pentapeptide of human C5a (MQLGR, residues 70–74) has been described by Chenoweth *et al.* (1979).

The six pentapeptides are compared functionally to the C3a peptide analog LGLAR in Table 2. Activities of the octapeptide analogs of C3a and C4a are compared to the native factors in Table 3. Since the COOH-terminal C4a pentapeptide differs from the C3a pentapeptide at two residue positions, the effects of each replacement on the inflammatory activities were separately examined.

Two synthetic pentapeptide analogs of C3a (73–77) with substitutions at position 76 (LGLSR and LGLQR) were much less active than two analogs with replacements at position 73 (VGLAR and AGLAR). Peptide LGLQR, is only 3% as active as the reference C3a peptide LGLAR in the ileal contraction assay (Table 2). Although this glutamine-76 analog qualitatively mimicked the activity of natural C3a and C4a *in vivo*, it required approximately a 20-fold greater amount of LGLQR than LGLAR to elicit the same permeability response. The serine-76 analog LGLSR gave a response of only 0.5–1.0% of LGLAR in the *in vitro* muscle assay and 5–6% in the *in vivo* skin assay (Table 2). Substitution of either glutamine or serine for alanine-76 markedly diminished the activi-

Table 1. Chemical characterization of eight synthetic peptides related to human C3a and C4a

Peptide code	Amino acid Sequence[a] 70 71 72 73 74 75 76 77	Amino acid composition Gln	Gly	Ala	Leu	Arg	Other	Thin-layer chromatography[b] A	B	C	Reverse-phase chromatography[c] t_r, min
LGLAR	Leu-Gly-Leu-Ala-Arg		0.98	0.99	1.99	1.05		0.29	0.51	0.68	4.3
AGLAR	Ala-Gly-Leu-Ala-Arg		0.99	2.00	0.98	1.00		0.24	0.42	0.64	3.0
VGLAR	Val-Gly-Leu-Ala-Arg		0.98	0.98	0.98	1.05	Val,1.02	0.34	0.50	0.66	3.4
LGLQR	Leu-Gly-Leu-Gln-Arg	1.02	0.99		2.00	0.99		0.12	0.48	0.54	4.3
LGLSR	Leu-Gly-Leu-Ser-Arg		0.99		1.98	1.03	Ser,0.93	0.33	0.47	0.69	3.7
AGLQR	Ala-Gly-Leu-Gln-Arg	0.97	1.03	0.99	1.01	1.00		0.16	0.43	0.55	3.2
AAAAGLQR	Ala-Ala-Ala-Ala-Gly-Leu-Gln-Arg	0.99	1.02	4.03	0.97	0.98		0.02	0.29	0.50	3.6
KGQAGLQR	Lys-Gly-Gln-Ala-Gly-Leu-Gln-Arg	1.96	2.08	1.04	0.96	0.98	Lys,0.98	0.10	0.12	0.35	2.6

[a]LGLAR is the COOH-terminal sequence of C3a, AGLQR and KGQAGLQR are COOH-terminal sequences of C4a, and VGLAR is the COOH-terminal sequence of bovine C4a. The other three pentapeptides are analogs of C3a/C4a. Underlined residues differ from the C3a sequence.

[b]R_f values for three solvent systems (by volume): A, 4:1:1 1-butanol/acetic acid/water; B, 1:1:1:1 1-butanol/ethyl acetate/acetic acid/water; C, 1:1:1 1-butanol/acetic acid/water. Each peptide showed only a single spot in each of these three solvent systems.

[c]A μBondpak C_{18} reverse-phase column (0.39 × 30 cm) was eluted isocratically at 4 ml/min with 0.1 percent acetic acid containing 25% methanol.

Table 2. Relative inflammatory activities of pentapeptides that mimic or are analogs to the COOH-terminal sequences of human C3a and C4a

Peptide structures	Human anaphylatoxin fragment	Relative activity: Smooth Muscle Contraction[a]	Relative activity: Skin wheal[b]
73 74 75 76 77			
Leu-Gly-Leu-Ala-Arg	C3a-(73-77)	100	100
Val-Gly-Leu-Ala-Arg		85-95	90-100
Ala-Gly-Leu-Ala-Arg		17-25	10-15
Leu-Gly-Leu-Ser-Arg		0.5-1.0	5-6
Leu-Gly-Leu-Gln-Arg		3.0-3.5	5-6
Ala-Gly-Leu-Gln-Arg	C4a-(73-77)	0.05-0.07	0.1

[a]Peptide LGLAR contracts guinea-pig ileal smooth muscle at a concentration of $3\text{-}4 \times 10^{-6}$ *M* in the 7 ml tissue bath.

[b]An 8 mm guinea-pig skin wheal is produced by 4×10^{-9} mole of the C3a pentapeptide LGLAR.

ties of the resultant peptide. Therefore, it is not surprising that the alanine-73, glutamine-76 analog AGLQR, which is the COOH-terminal pentapeptide of human C4a, exhibits only 0.05% of the activity of LGLAR.

Substitution of leucine-73 in LGLAR by alanine furnished the peptide AGLAR, which exhibited approximately 5–10 fold lower activity than LGLAR (Table 2). Since human C4a is only 1% as active as C3a, the substitutions of alanine for leucine-73 and glutamine for alanine-76 both appear to decrease the inflammatory activities of C4a relative to C3a. Interestingly, the bovine C4a pentapeptide VGLAR is equally as active as the C3a pentapeptide LGLAR, which suggests that bovine C4a may be considerably more active than human C4a.

Injection of 75–100 nmole of LGLQR into human forearm skin produced an immediate wheal and flare accompanied by slight itching. The area of the wheal and flare was maximal in 10–15 min and had virtually disappeared in 60 min. During the first 15 min, the area close to the wheal was numb to the touch. Furthermore, 2–5 μmole of the C4a octapeptide KGQAGLQR or 10–20 nmole of native C4a produced a similar effect and an additional stinging sensation.

Since the C4a pentapeptide AGLQR is only marginally active, less active compared to C4a than the C3a pentapeptide LGLAR was when compared to C3a, we examined several longer peptide analogs of the C4a sequence. Both the COOH-terminal C3a-(70–77) octapeptide, ASHLGLAR and a trialanyl analog, AAALG-

Table 3. Inflammatory activities of synthetic C3a and C4a peptides and analogs compared to the natural anaphylatoxins

	Smooth muscle contraction[a]: Effective doses[a] (molar)	Smooth muscle contraction[a]: Relative activity	Skin wheal[b]: Effective doses (nmol)	Skin wheal[b]: Relative activity
Native human C3a residues (1-77)	$6\text{-}9 \times 10^{-9}$	(100)	0.1-0.2	(100)
70 77 Ala-Ser-His-Leu-Gly-Leu-Ala-Arg	$9\text{-}12 \times 10^{-7}$	0.7	5-6	3
Ala-Ala-Ala-Leu-Gly-Leu-Ala-Arg	$5\text{-}7 \times 10^{-7}$	1.3	5-6	3
Native human C4a residues (1-77)	$1\text{-}2 \times 10^{-7}$	(100)	10-20	(100)
70 77 Lys-Gly-Gln-Ala-Gly-Leu-Gln-Arg	$1.0\text{-}1.5 \times 10^{-3}$	0.1	2500-5000	0.3-0.6
Ala-Ala-Ala-Ala-Gly-Leu-Gln-Arg	$1.0\text{-}1.5 \times 10^{-4}$	1.0	2500-5000	0.3-0.6

[a]Minimum concentration of factor required to elicit full contraction of guinea-pig ileal strip in a 1.5 ml bath.

[b]Quantity of factor that induces a wheal 8–11 mm in diameter when injected intradermally in guinea-pig skin.

EXHIBIT 5

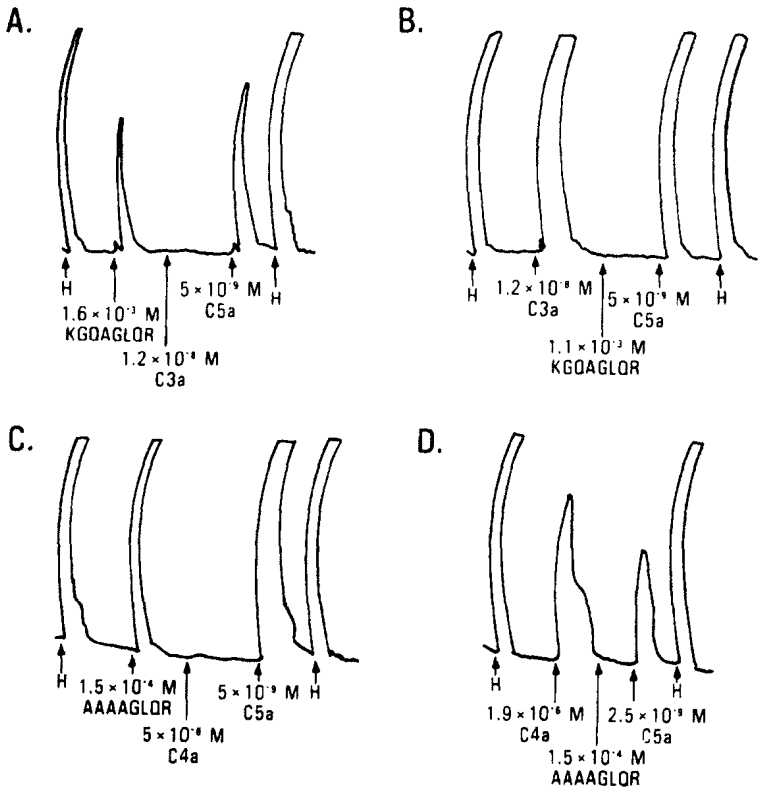

Fig. 1. Effect of synthetic C4a peptide KGQAGLQR and trialanyl analog AAAAGLQR on contraction of guinea-pig ileum. A fresh strip of relaxed guinea-pig ileum approximately 2.5 cm in length was challenged with the factor in a 1.5 ml organ bath. A separate muscle strip was used for each experiment. Contraction is indicated by a vertical pen deflection. Panel A, the peptide KGQAGLQR induced a contraction and desensitized the muscle to human C3a but not to human C5a or histamine. Panel B, a muscle strip stimulated by human C3a was desensitized to subsequent stimulation by peptide KGQAGLQR but not to C5a. Panel C, the peptide AAAAGLQR desensitized the muscle strip to human C4a but not to C5a. Panel D, human C4a desensitized the muscle strip to peptide AAAAGLQR.

LAR, were assayed for comparison to the corresponding octapeptide analogs of C4a (Table 3). The C4a octapeptide KGQAGLQR induced full contraction of an isolated guinea-pig ileal strip at a concentration of 1–1.5 × 10^{-3} *M*, which is a level 1000-fold higher than that required for C4a. Smooth muscle tissue exposed to KGQAGLQR was specifically desensitized to subsequent stimulation by either human C3a or C4a (not shown) but not to contraction by human C5a or histamine (Fig. 1A). Conversely, stimulation of a muscle strip by C3a or C4a caused desensitization to subsequent stimulation by KGQAGLQR (Fig. 1B). We also observed cross-tachyphylaxis between the trialanyl C4a analog AAAAGLQR and C4a (Fig. 1C and 1D). Thus the actions of these synthetic peptides and natural C3a and C4a are all focused at a common cellular receptor.

Finally, repeated application of AAAAGLQR or KGQAGLQR to ileal muscle strips also desensitized the tissue. Evidence for cross- and self-tachyphylaxis by the C3a/C4a peptides on ileal tissue agrees with the characteristic desensitization exhibited by the natural anaphylatoxins. The C3a- (70–77) octapeptide ASHLGLAR is approx. 1% as active as C3a and the trialanyl analog AAALGLAR is 2% as active as C3a for inducing smooth muscle contraction. Both of the C3a octapeptides tested exhibit about 3% of the activity of C3a in the *in vivo* skin test. The C4a-(70–77) octapeptide KGQAGLQR was only three times as active as the C4a-(73–77) pentapeptide AGLQR and only 0.1% as active as natural C4a. Surprisingly, the trialanyl C4a analog AAAAGLQR was 10 times more active than the true C4a octapeptide KGQAGLQR. At 1.5 × 10^{-4} *M*

AAAAGLQR desensitizes the ileal tissue to both C3a and C4a.

DISCUSSION

Synthetic peptides based on the COOH-terminal sequence of human C3a exhibit both the specificity and biological activities of the natural anaphylatoxin (Hugli & Erickson, 1977). The C3a pentapeptide LGLAR is about 0.2% as potent on a molar basis as native C3a for contracting ileal smooth muscle (Caporale *et al.*, 1977, 1979). Longer synthetic C3a peptides containing 8 and 13 residues are approximately 12 and 30 times more active, respectively, than is the pentapeptide. In contrast, the COOH-terminal tetrapeptide GLAR lacking leucine-73 is 40 times less active than the pentapeptide. Thus the synthetic pentapeptide LGLAR has proven to be a convenient model of the C3a active site and useful in examining the biological activities of C3a anaphylatoxin.

Effects on smooth muscle activity of replacing one residue by another in the C3a pentapeptide have revealed several important structural features concerning this active site model. Leucine-73 can be replaced by several other large hydrophobic residues with little decrease in activity. However, leucine-75 cannot be replaced by any other amino acid without substantial loss in activity (Unson *et al.*, 1979). Furthermore, both the positively charged guanidinium group and the negatively charged carboxylate group of the COOH-terminal arginine are essential for activity (Erickson *et al.*, 1981).

In the course of replacing alanine-76 of the C3a pentapeptide LGLAR, to more closely mimic the C4a sequence AGLQR, we found that the glutamine-76 analog LGLQR was only 2–3% as active as LGLAR in the ileal contraction assay and 5–6% as active in the *in vivo* skin assay. The serine-76 analog LGLSR was even less active for inducing smooth muscle contraction. Both of these analogs appear to act through the cellular C3a/C4a receptor because each rendered the ileal tissue tachyphylactic to C3a (data not shown). The substantial loss in potency upon replacing the methyl side chain of alanine-76 by the hydroxymethyl side chain of serine is probably due to the increased size of the side chain. Although the seryl side chain is only one oxygen atom larger than the alanyl side chain, in aqueous media the hydroxyl group of serine is surrounded by a hydration sphere that significantly increases its effective size.

Substitution of alanine-76 by glutamine in the synthetic model peptides was indicated by the report of Gorski *et al.* (1979*b*) that the COOH-terminal sequence of human C4a was . . .–Leu–Gln–Arg–OH. Knowing the effect on activity of substituting the seryl side chain for alanine, we can only conclude that the glutamyl side chain also diminishes activity of the pentapeptide because of its bulk. More recently, the entire sequence of C4a has been determined (Moon *et al.*, 1981). Inspection of the COOH-terminal pentapeptide of human C4a, AGLQR, shows that it differs from the C3a active-site pentapeptide at both positions 73 and 76, indicating that C4a may be a significantly less potent factor than C3a.

As shown in Fig. 1, the C4a octapeptide KGQAGLQR exhibited cross-tachyphylaxis towards human C3a, did not desensitize the ileal tissue towards contraction induced by either human C5a or histamine, and was only 0.1% as active on a molar basis as natural C4a in the ileal contraction assay. The C3a octapeptide is about 1% as active as natural C3a. Since human C4a is only 1% as active on a molar basis as human C3a (Gorski *et al.*, 1979*b*), the C4a peptides were expected to be relatively inactive compared to C3a and the C3a peptides. Nevertheless, a structural and functional similarity exists between the well-defined, active site pentapeptide of C3a, LGLAR (Erickson *et al.*, 1981) and the COOH-terminal pentapeptide AGLQR of human C4a. Based on these similarities we conclude that AGLQR comprises the essential active site of the C4a molecule. The preceding 70 residues in human C4a serve quantitatively to increase, by about 300-fold, the intrinsic activity of the pentapeptide AGLQR, but they do not qualitatively alter the anaphylatoxin activity expressed by the five COOH-terminal residues.

The C4a synthetic peptides mimic the anaphylatoxin activity of human C3a *in vivo* as well as *in vitro*. As shown in Fig. 2, peptides AGLAR and LGLQR were about 6–10% as potent as the C3a peptide LGLAR for eliciting a visible wheal upon injection into the human forearm. Although this assay is less precise than the *in vitro* tissue contraction assay, it nevertheless demonstrates that a relatively small quantity (0.1 μmole) of either of the C4a

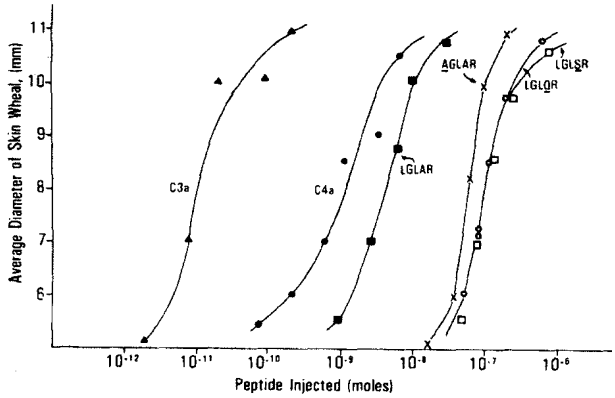

Fig. 2. *In vivo* response of human skin to increasing doses of synthetic C3a and C4a peptides analogs. The visible wheal at the injection site was recorded 15 min after a 50-μl sample containing the appropriate peptide was injected subcutaneously. The skin response to native human C3a (▲) and human C4a (●) are presented for comparison. Virtually identical results were obtained from guinea-pig skin (see Tables 2 and 3).

active-site analog peptides produces a distinct inflammatory skin reaction. Unfortunately, the C4a peptides AGLQR and KGQAGLQR were active only when large quantities were injected (curves not shown). Interestingly, the trialanyl C4a analog AAAAGLQR was active in the μmole range.

The active-site C3a peptide contains an alanine in the penultimate position, whereas the active-site C4a peptide contains glutamine. The replacement of alanine by glutamine requires a minimum of two base changes between the respective codons. One of two residues that could serve as a direct genetic intermediate between alanine and glutamine is proline. An alanine codon may be converted into a proline codon by replacement of the first base guanine by cytosine. Similarly, the glutamine codon is converted into a proline codon by replacement of the second base adenine by cytosine. The proline analog LGLPR, a model of a possible evolutionary intermediate between human C3a and C4a, was equally as active both in the *in vivo* skin assay and in the more precise ileal contraction assay as the C3a/C4a pentapeptide analog LGLQR (data not shown). Consequently, mutation of the first base in the C3a alanine-76 codon from guanine to cytosine would furnish an intermediate C3a/C4a anaphylatoxin with reduced activity. Subsequent mutation of the second base of the resulting proline-76 codon from cytosine to adenine would produce the COOH-terminal pentapeptide LGLQR akin to C4a, with little change in activity from the intermediate polypeptide containing proline at position 76. This scenario would have to be reversed if C4 evolved from an ancestral C3 gene.

Not all amino acid substitutions for glutamine-76 in C4a will result in increased anaphylatoxin activity. For example, replacement by serine, which requires one base change from proline or alanine, produces the analog LGLSR, which is only about 20–30% as active as LGLQR in the ileal contraction assay. All of the pentapeptides in the C3a/C4a series were active at some level and were cross-tachyphylactic for C3a but not for C5a. Interestingly, the COOH-terminal pentapeptide of C5a, MQLGR, was inactive when tested at levels that exceeded those examined in the present study (Chenoweth *et al.*, 1979).

It should be noted that intradermal injection of 1 μmole (540 μg) of the C4a pentapeptide AGLQR produced a transient (1–2 min) mild stinging sensation comparable to that induced by a mosquito bite; when similar levels of LGLSR and LGLQR were injected, they produced only an itching sensation. This level of AGLQR, however failed to produce a visible wheal. A comparable stinging sensation was observed by one of us (TEH) when 10 nmole (90 μg) of human C4a were injected into the skin on his forearm during both these and previous experiments (Gorski *et al.*, 1979*b*). This qualitative observation was not reported previously because relatively few injections of C4a had been performed at the time and because this response might have been due to the presence of salts or other experimental variables.

Production of a similar stinging sensation after injection of the synthetic C4a pentapeptide, however, supports the association of stinging and itching with the factor C4a. Recent evidence that anaphylatoxins may stimulate prostaglandin synthesis (Stimler *et al.*, 1983) may explain the mildly painful effects of C4a. These same sensations are seldom elicited when elevated levels of synthetic C3a peptides are injected, such as those used to obtain maximal wheal and flare responses in Fig. 2. In contrast, no stinging and itching was ever sensed upon injection of even greater doses of the C5a pentapeptide MQLGR. This ability of anaphylatoxins to produce mild pain or itching has not been previously observed with C3a or C5a. It should be emphasized, however, that quantities of intact C3a or C5a larger than a few nanomoles have never been injected into human subjects.

Donaldson *et al.* (1977) have ascribed a kinin-like activity present in the plasma of individuals with hereditary angioneurotic edema (HANE) to a fragment derived from complement C2. Since production of maximal kinin-like activity required the presence of not only the enzyme C1s and C2 but also component C4, a fragment of C4 might also be involved. The biological responses due to human C4a and the C4a active-site peptides are very similar to the kinin-like activity attributed to a C2 fragment. Complement activation proceeds rapidly in HANE plasma and in other plasma samples deficient in C1 inactivator and therefore C4a may be formed at a faster rate than it can be inactivated by serum carboxypeptidase N (anaphylatoxin inactivator; EC 3.4.12.7). Consistent with this possibility is the fact that as much as 3 nmole of C4a could be generated in 1 ml of plasma and, as shown in Fig. 2, less than 1 nmole of C4a produces a visible wheal in human skin. In addition, the human skin response described by Klemperer *et al.* (1967) on injection of C1s is quite similar to that observed after injection of either C4a or the C4a peptide analogs. Therefore, the edema observed in HANE patients may not only result from generation of bradykinin (Curd *et al.*, 1982) but also from production of C4a anaphylatoxin. In light of the present observations, patients with HANE should be re-examined for the presence of active C4a. It seems fair to hypothesize that anaphylatoxins along with bradykinin could promote the clinical expression of angioneurotic edema.

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Exhibit 6

EXHIBIT 6

CUTTING EDGE

Cutting Edge: Human Anaphylatoxin C4a Is a Potent Agonist of the Guinea Pig But Not the Human C3a Receptor[1,2]

Stefan Lienenklaus,* Robert S. Ames,‡ Mark A. Tornetta,‡ Henry M. Sarau,† James J. Foley,† Torsten Crass,* Bettina Sohns,* Ute Raffetseder,* Melanie Grove,* Anja Hölzer,* Andreas Klos,* Jörg Köhl,* and Wilfried Bautsch[3]*

The interaction of human anaphylatoxin C4a with the guinea pig (gp) and human (hu) C3a receptors (C3aR) was analyzed using human rC4a, which exhibited C4a-specific activity on guinea pig platelets. A gpC3aR of 475 residues with a large second extracellular loop and a peptide sequence ~60% identical to the huC3aR was isolated from a genomic DNA library and found to be expressed in guinea pig heart, lung, and spleen. HEK-293 cells cotransfected with this clone, and a cDNA encoding Gα-16 specifically bound (K_d = 1.6 ± 0.7 nM) and responded functionally to C3a with an intracellular calcium mobilization (ED_{50} = 0.18 ± 0.02 nM). Human rC4a weakly bound to both the hu- and gpC3aR (IC_{50} > 1 μM). However, only HEK-293 cells expressing the gpC3aR responded functionally to rC4a (ED_{50} = 8.7 ± 0.52 nM), while cells expressing the huC3aR did not ($c \leq 1$ μM). Thus, through an interaction with the C3aR, huC4a may elicit anaphylatoxic effects in guinea pigs but not in man. ***The Journal of Immunology,*** **1998, 161: 2089–2093.**

The anaphylatoxins C3a, C4a, and C5a are small proteins of 74 to 77 residues that are generated in an inflammatory reaction by proteolytic cleavage from the complement components C3, C4, and C5, respectively. C3a and C5a are important chemotactic proinflammatory molecules, mediating smooth muscle contraction, increase in vascular permeability, and various cell activation and granule secretion reactions (for an overview, see Refs. 1 and 2).

Human C4a, first described in 1979, is regarded as the third anaphylatoxin (3) due to its structural similarity to C3a and C5a, its dependence on a carboxyl-terminal arginine residue for biologic activity, and its proinflammatory properties in guinea pigs. Although less active than C3a and C5a, in guinea pigs, human C4a induces smooth muscle contraction, increases vascular permeability (3), and induces granule secretion from platelets and O_2^- generation in macrophages (4, 5).

These biologic effects of C4a are subject to low dose desensitization (tachyphylaxis). Preincubation with substimulatory concentrations of C4a abrogates the functional response toward a subsequent 100% stimulus. In addition, cross-desensitization in guinea pig ileal contraction assays was observed between C4a and C3a, but not between C4a and C5a (3, 4). Based on these observations, C3a and C4a are thought to act on a common receptor. This view, however, has recently been questioned by Murakami and coworkers, who failed to detect desensitization of C3a-induced responses after pretreatment of guinea pig macrophages with human C4a (5). Furthermore, C4a did not inhibit binding of ^{125}I-labeled C3a to guinea pig macrophages (5). These findings would indicate distinct and separate receptors for C3a and C4a in guinea pigs.

In man, convincing evidence for anaphylatoxic effects of human C4a is still missing. C4a has been reported to inhibit C3a-induced chemotaxis of macrophages (6), although at extremely low concentrations (10^{-16} M!). Also, C4a and C4a analogue synthetic peptides lead to a dose-dependent wheal and flare generation when injected into human skin (3, 7), although no negative controls were included in these experiments. Evidence for C4a effects in species other than man or guinea pig has not been reported.

Recently, the C3aR from man (huC3aR)[4] (8–10) and mouse (11, 12) have been cloned. Stably transfected cell lines expressing these receptors and human neutrophils have been tested for functional response towards serum-purified human C4a and found to be completely unresponsive (11, 13). This would suggest the existence of a separate C4a receptor in man and mouse, on the premise that C4a is an anaphylatoxin in these species as well. However, no

*Institute of Medical Microbiology, Hannover Medical School, Hannover, Germany; and Departments of †Pulmonary Pharmacology and ‡Molecular Biology, SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406

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[3] Address correspondence and reprint requests to Dr. Wilfried Bautsch, Institute of Medical Microbiology, Hannover Medical School, Carl-Neuberg-Strasse 1, D-30623 Hannover, Germany. E-mail address: Bautsch.Wilfried@MH-Hannover.de.

[4] Abbreviations used in this paper: huC3aR, human C3a receptor; gp, guinea pig (i.e., gpC3aR); FLIPR, fluorometric imaging plate reader; *c*, concentration.

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positive control demonstrating biologic activity of the C4a preparation used in these experiments was presented.

Highly purified human C4a is difficult to prepare and, due to the high C4a concentrations required in most test systems, even trace contamination with other biologically active molecules, especially C3a and C5a, may jeopardize the experiments, as has been reported in one of the above-mentioned investigations for serum-purified C4a (13). This possible contamination may well be the cause for some of the discrepant results presented above. Using human rC4a and cells functionally expressing a cloned guinea pig (gp) or huC3aR, we provide evidence that C4a functions as an agonist of the gpC3aR but not the huC3aR.

Materials and Methods

Materials

Human C3a was obtained from Advanced Research Technologies (San Diego, CA), ^{125}I-labeled human C3a from NEN-DuPont (Boston, MA), and rC5a from Sigma (St. Louis, MO). The C3a carboxyl-terminal analogue synthetic peptide (WWGKKYRASKLGLAR, (W63,W64)C3a(63-77) (14)) was obtained from Bachem (King of Prussia, PA), and the C3a synthetic peptide P117 and control peptide P251 were prepared as described (9). Oligonucleotides were obtained from MWG Biotech (Ebersberg, Germany). N-terminal hexahistidine-tagged rC4a or rC4a with an N-terminal methionine residue was expressed in *Escherichia coli* and purified to homogeneity. The binding and functional activities of these two preparations of rC4a were equivalent.

Guinea pig platelet release assay

Functional characterization of rC4a was performed in a guinea pig ATP release assay, as described (15). Guinea pigs from strains C2BB/R$^+$ (C3aR positive) and C2BB/R$^-$ (C3aR-negative) from our own breeding colonies were used as platelet donors (16). Desensitization was measured by preincubation of the platelets with the deactivating stimulus (a concentration leading to <10% ATP release, determined empirically at the beginning of each experiment) for 5 min at 37°C and subsequent addition of a 100% stimulus of either C3a (c = 10 nM), rC4a (c = 4 μM), or rC5a (c = 250 nM).

Cloning of the guinea pig C3aR

A partial DNA sequence of the gpC3aR was obtained by PCR amplification of genomic DNA using oligonucleotide primers derived from regions conserved between the human and mouse C3aR sequences (8–12). This fragment was used to screen a guinea pig genomic DNA library in λFIXII (Stratagene, La Jolla, CA). A λ clone containing a genomic insert, which encoded the gpC3aR, was identified. The open reading frame of this clone plus an extra 48 bp of genomic DNA sequence at the 3′ end was subcloned into pcDNA3 (Invitrogen, San Diego, CA) and designated pSL94.

Receptor characterization

Competitive binding assays were performed essentially as described (9). A microtiter plate-based calcium mobilization assay, utilizing a fluorometric imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA), was used for the functional characterization of HEK-293 cells transiently expressing either the gpC3aR or huC3aR and Gα-16 (17). Briefly, cells were transfected using Lipofectamine Plus reagent (Life Technologies, Gaithersburg, MD), and the following day they were plated in poly-D-lysine-coated 96-well black/clear plates (Becton Dickinson, Bedford, MA). After 18 to 24 h, the medium was aspirated from each well, and the cells were loaded with Fluo-3-AM (Molecular Probes, Eugene, OR). At initiation of the assay, fluorescence is read once every second for 60 s and then every 3 s for the following 60 s. Agonist was added at 10 s, and the maximal fluorescence count above background after addition was used to define maximal activity for that concentration of agonist. FLIPR software normalizes fluorescence readings to give equivalent readings for all wells at zero time.

Results and Discussion

Generation and characterization of rC4a

To avoid the problem of contamination in serum-purified human C4a preparations, we expressed rC4a. As shown in Figure 1*A*, human rC4a was approximately three orders of magnitude less potent in a guinea pig ATP release assay than C3a, but induced ATP release at high nanomolar concentrations ($ED_{50} \approx 400$ nM), comparable with the ED_{50} of 600 nM determined previously for serum-purified C4a in the serotonin release assay (4). Furthermore, in this assay, complete cross-tachyphylaxis between human C3a and rC4a, but not C5a, was observed (Fig. 1*B*). In addition, platelets of the C3aR-negative guinea pig strain C2BB/R$^-$, an inbred strain of our own breeding colonies with a functional C3aR deficiency (16), did not respond to either C3a or rC4a (data not shown). These data demonstrate the functional activity of the rC4a and confirm previous data on guinea pig cells showing cross-tachyphylaxis between C3a and C4a (3, 4). The simplest explanation of these data would be that C3a and C4a act through the same receptor. However, postreceptor mechanisms may account for the observed cross-tachphylaxis, as in the case of the FMLP-R and C5aR (18). We, therefore, set out to clone a C4a-binding C3aR from the guinea pig.

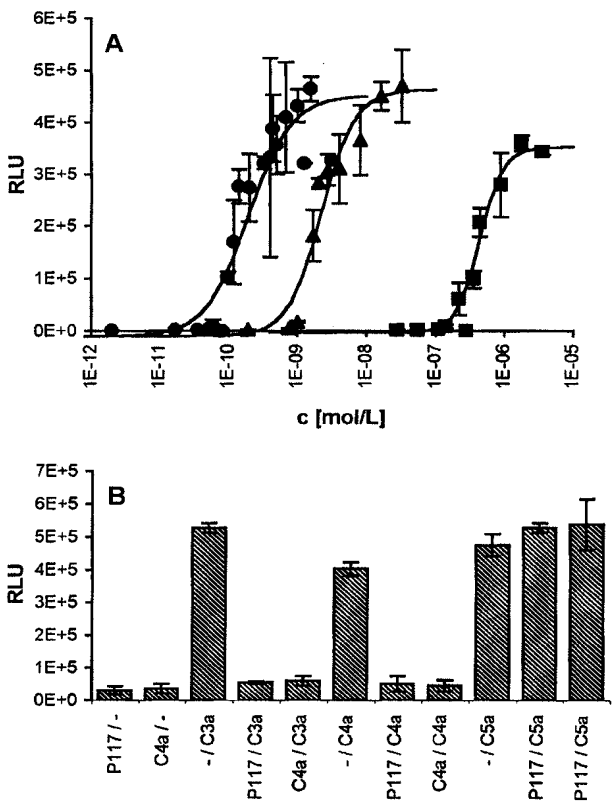

FIGURE 1. Functional characterization of rC4a in the guinea pig platelet ATP release assay. *A*, Activation curves of C3a (circles; $ED_{50} = 4.9 \pm 1.3 \times 10^{-10}$ M), rC4a (squares; $ED_{50} = 4.1 \pm 0.6 \times 10^{-7}$ M) and the C3a analogue synthetic peptide P117 (triangles) (n = 3) (9). *B*, Desensitization of rC4a and C3a, but not rC5a, responses after preincubation of the platelets with substimulatory concentrations of rC4a or the C3a analogue synthetic peptide P117, respectively, and subsequent addition of C3a (10 nM), rC4a (4 μM), or rC5a (250 nM). RLU, relative light units; "X/Y", deactivating stimulus/100% stimulus.

Cloning and characterization of gpC3aR

The coding regions of many G protein-coupled receptors, including the human C3aR (19), are not interrupted by introns. Therefore, we cloned the gpC3aR directly from a genomic DNA library.

```
1-ATGGAGTCTTCCTCTGCTGAAACCAACTCAACTGGCCTACACTTAGAACCTCAGTATCAACCCGAAACAATTCTGGCCATGGCCATCCTA
  MetGluSerSerSerAlaGluThrAsnSerThrGlyLeuHisLeuGluProGlnTyrGlnProGluThrIleLeuAlaMetAlaIleLeu

91-GGCCTCACTTTTGTTCTGGGGTTGCCCGGCAATGGGCTGGTGCTGTGGGTGGCTGGCCTGAAGATGCGGCGCACAGTGAACACAGTTTGG
  GlyLeuThrPheValLeuGlyLeuProGlyAsnGlyLeuValLeuTrpValAlaGlyLeuLysMetArgArgThrValAsnThrValTrp

181-TTTCTTCACCTCACCGTGGCAGACTTCGTCTGCTGCCTCTCCTTGCCTTTCTCCATGGCTCATTTGGCTCTCCGAGGGTACTGGCCCTAT
  PheLeuHisLeuThrValAlaAspPheValCysCysLeuSerLeuProPheSerMetAlaHisLeuAlaLeuArgGlyTyrTrpProTyr

271-GGCGAGATACTCTGCAAGTTTATCCCAACTGTCATCATCTTCAACATGTTCGCTAGCGTTTTCCTGCTTACTGCTATTAGCCTGGACCGG
  GlyGluIleLeuCysLysPheIleProThrValIleIlePheAsnMetPheAlaSerValPheLeuLeuThrAlaIleSerLeuAspArg

361-TGCTTGATGGTACTCAAGCCAATCTGGTGCCAGAATCATCGCAATGTGAGAACAGCCTGCATTATCTGTGGATGCATTTGGCTGGTGGCT
  CysLeuMetValLeuLysProIleTrpCysGlnAsnHisArgAsnValArgThrAlaCysIleIleCysGlyCysIleTrpLeuValAla

451-TTTGTCCTGTGTATACCTGTGTTTGTGTACCGAGAAACGTTCACTCTAGAAAACCACACTATCTGTACCTATAATTTTAGTCCCGGATCA
  PheValLeuCysIleProValPheValTyrArgGluThrPheThrLeuGluAsnHisThrIleCysThrTyrAsnPheSerProGlySer

541-TTCGACTATTTAGACTACGCCTATGACCGAGATGCATGGGGCTATGGAACTCCTGACCCCATTGTCCAGCTGCCTGGAGAAATGGAACAT
  PheAspTyrLeuAspTyrAlaTyrAspArgAspAlaTrpGlyTyrGlyThrProAspProIleValGlnLeuProGlyGluMetGluHis

631-AGATCAGATCCTTCTTCTTTCCAAACACAGGATGGCCCTTGGTCAGTCACCACTACCCTTTATTCTCAAACATCTCAAAGACCTTCTGAA
  ArgSerAspProSerSerPheGlnThrGlnAspGlyProTrpSerValThrThrThrLeuTyrSerGlnThrSerGlnArgProSerGlu

721-GATTCATTTCATATGGATTCTGCAAAATTATCTGGTCAAGGTAAATATGTTGATGTAGTCTTACCCACAAACCTCTGTGGGCTTCCTATG
  AspSerPheHisMetAspSerAlaLysLeuSerGlyGlnGlyLysTyrValAspValValLeuProThrAsnLeuCysGlyLeuProMet

811-GAAGAGAACAGAACTAACACATTGCATAATGCTGCTTTTCTCTCCTCTGATTTAGATGTCTCCAATGCAACCCAAAAGTGCTTATCCACA
  GluGluAsnArgThrAsnThrLeuHisAsnAlaAlaPheLeuSerSerAspLeuAspValSerAsnAlaThrGlnLysCysLeuSerThr

901-CCTGAGCCACCACAAGACTTCTGGGATGATTTAAGCCCGTTCACACATGAATATCGAACACCAAGACTTTTAAAAGTAATAACCTTCACA
  ProGluProProGlnAspPheTrpAspAspLeuSerProPheThrHisGluTyrArgThrProArgLeuLeuLysValIleThrPheThr

991-AGACTAGTGGTGGGTTTCCTGCTGCCTATGATCATCATGGTGGCCTGTTACACTCTCATCATCTTCCGAATGCGACGGGTCCGCGTTGTC
  ArgLeuValValGlyPheLeuLeuProMetIleIleMetValAlaCysTyrThrLeuIleIlePheArgMetArgArgValArgValVal

1081-AAGTCTTGGAACAAAGCCCTTCACCTGGCCATGGTGGTGGTGACCATCTTTCTTATCTGCTGGGCTCCATATCATGTTTTTGGAGTCCTC
  LysSerTrpAsnLysAlaLeuHisLeuAlaMetValValValThrIlePheLeuIleCysTrpAlaProTyrHisValPheGlyValLeu

1171-ATATTGTTTATTAACCCGGAAAGTCGCGTTGGGGCAGCTCTGTTGTCTTGGGACCACGTGTCCATTGCTCTTGCATCTGCTAACAGTTGC
  IleLeuPheIleAsnProGluSerArgValGlyAlaAlaLeuLeuSerTrpAspHisValSerIleAlaLeuAlaSerAlaAsnSerCys

1261-TTTAATCCTTTCCTTTACGCCCTCTTGGGGAGAGATCTTAGGAAGCGAGTGAGACAGTCCATGAAGGGCATTCTGGAGGCAGCCTTCTCT
  PheAsnProPheLeuTyrAlaLeuLeuGlyArgAspLeuArgLysArgValArgGlnSerMetLysGlyIleLeuGluAlaAlaPheSer

1351-GAGGATATCAGCAAGTCTACCAGTTTTATCCAAGCCAAAGCCTTTTCAGAAAAACACAGCTTGAGTACAAATGTGTAA
  GluAspIleSerLysSerThrSerPheIleGlnAlaLysAlaPheSerGluLysHisSerLeuSerThrAsnVal---
```

FIGURE 2. Nucleotide and deduced amino acid sequence of the cloned gpC3aR. The position of the four putative *N*-glycosylation sites is indicated in bold, the putative seven-transmembrane regions are underlined. The GenBank accession number for this nucleotide sequence is AJ006402.

Using primer combinations conserved in the C3aR sequences of man (8–10) and mouse (11, 12), a genomic DNA fragment was amplified with high homology to the huC3aR. Using this fragment as a probe to screen a guinea pig library, a genomic λ clone was isolated with an open reading frame of 1428 bp, which encoded a protein of 475 residues with a calculated molecular mass of 53,570 Da and four potential *N*-glycosylation sites (Fig. 2). The sequence encodes a G protein-coupled receptor with seven hydrophobic transmembrane domains, a large second extracellular loop of 165 amino acid residues, and a high homology to the C3aR sequences of man, mouse, and rat (8–12, 20). However, only half of the residues are conserved in all four known C3aR sequences (240/475 = 50.5%), and only 37 of the 165 residues in the second extracellular loop (22.4%) in the gpC3aR are found at the same position in the other C3aR sequences. The peptide sequence of this gpC3aR was disclosed in a recently published book (2); however, the nucleotide sequence has not been published nor does it appear in GenBank, and no functional or binding data have been presented. It is unlikely that this receptor is a pseudogene, since by RT-PCR, expression of this transcript was detected in guinea pig heart, lung, and spleen RNA (data not shown).

HEK-293 cells transiently transfected with this receptor specifically bound human C3a. As shown in Figure 3, competitive displacement studies revealed the presence of a high affinity receptor for C3a with an apparent K_d of ~2 nM. As previously shown with the huC3aR, this binding was specific for C3a, because ^{125}I-labeled C3a could be displaced in a dose-dependent manner by the synthetic C3a analogue peptide P117, but not by the unrelated peptide P251 (9). In addition, supramicromolar concentrations of rC4a ($IC_{50} > 1$ μM) were able to competitively displace radiolabeled C3a from both the guinea pig and huC3aR. Although human C4a is able to bind weakly to both the gpC3aR and huC3aR, the affinity of this interaction is three orders of magnitude lower than the affinity of C3a binding to the same receptors.

C4a mediates functional responses via the gpC3aR but not the huC3aR

To compare the functional activity of the gpC3aR and huC3aR, we cotransfected the cDNA for each receptor, together with a cDNA clone encoding Gα-16, into HEK-293 cells and tested for intracellular calcium mobilization in response to rC4a, rC5a, C3a, or a C3a analogue peptide. Previously, we reported that cotransfection of the huC3aR sequence with Gα-16 was necessary to render transfected HEK-293 cells responsive to C3a (9). As shown in Figure 4*A*, cells expressing the gpC3aR responded in a dose-dependent manner not only to C3a ($ED_{50} = 0.18 \pm 0.02$ nM) and the C3a peptide ($ED_{50} = 0.15 \pm 0.01$ nM), but also to rC4a ($ED_{50} =$

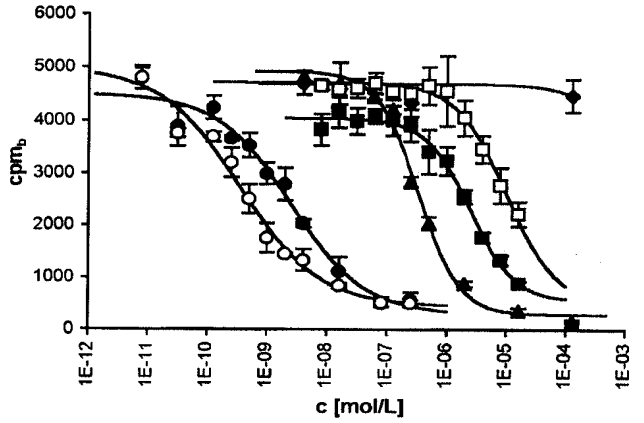

FIGURE 3. Competitive binding curves in HEK-293 cells transiently transfected with the gpC3aR (filled symbols) or huC3aR (open symbols), respectively. ^{125}I-labeled C3a was competitively displaced by increasing concentrations of C3a (circles), rC4a (squares), the synthetic C3a analogue peptide P117 (triangle), or the nonrelated synthetic peptide P251 (diamonds). C3a, K_d = 1.6 ± 0.9 nM (gpC3aR); K_d = 1.0 ± 0.9 nM (huC3aR); P117, IC_{50} = 1.9 ± 1.0 × 10^{-7} M (gpC3aR) ($n \geq 3$).

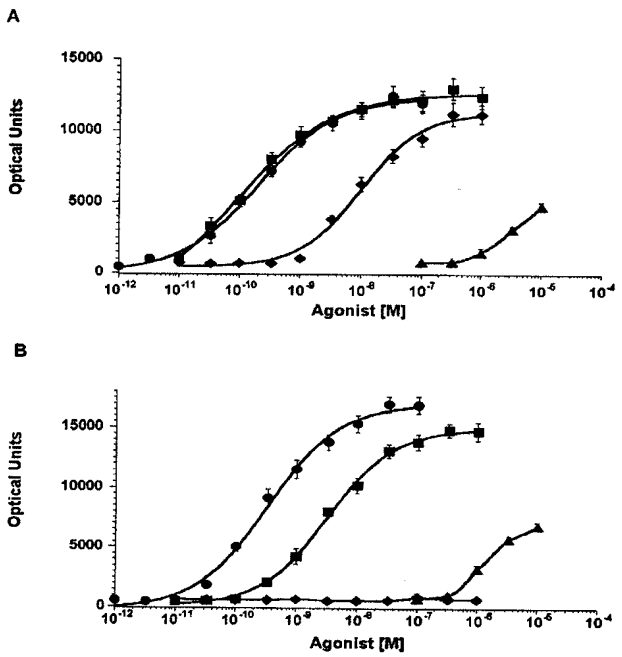

FIGURE 4. Calcium mobilization dose response curves of HEK-293 cells transiently expressing Gα-16 and the gpC3aR (*A*) or the huC3aR (*B*) to a stimulus of C3a (circles), (W63,W64)C3a(63-77) (squares), rC4a (diamonds), or rC5a (triangles). Concentration response curves for each agonist were run on six individual plates using FLIPR in two individual experiments. Values presented are the mean ± SEM.

8.7 ± 0.52 nM). Cells transfected with the cDNA for the gpC3aR or Gα-16 alone did not respond to any of the peptides tested (data not shown).

In marked contrast to the results obtained with rC4a on cells expressing the gpC3aR, cells transiently expressing the huC3aR did not respond to rC4a concentrations as high as 1 μM, but did respond to C3a (ED_{50} = 0.36 ± 0.07 nM) and the C3a peptide (ED_{50} = 3.1 ± 0.3 nM; Fig 4*B*). Previously, comparable results were obtained with stable cell lines expressing the cloned huC3aR (13). The gpC3aR and huC3aR responded differently to human rC4a as well as to the C3a synthetic peptide, which was virtually equipotent with C3a vs the gpC3aR but approximately an order of magnitude less potent than C3a vs the huC3aR. This may not be an unexpected result, as the biological assay originally used to characterize this "superagonist" C3a analogue peptide were all performed on guinea pig cells (14). Minimal activity was noted with rC5a on cells expressing either the gpC3aR or huC3aR, but only at supramicromolar concentrations (ED_{50} > 10 μM).

These data confirm that the receptor naturally expressed on guinea pig platelets is promiscuous with respect to human C3a and rC4a, and they extend these observations to demonstrate that both anaphylatoxins are also potent agonists of the cloned gpC3aR. In contrast, rC4a is not active on the huC3aR or mouse C3aR (this study and Ref. 13). The C3aR is a single-copy gene in man (19) and guinea pig (data not shown). Thus, human C4a cannot have a biologic effect in man that is mediated via the C3aR, whereas such a pathway apparently exists in guinea pigs. However, it remains to be determined whether gpC4a will also stimulate the gpC3aR.

Our data are difficult to reconcile with the findings of Murakami and coworkers who describe separate receptors for C3a and C4a on guinea pig macrophages (5), although these differences may result from the different reagents and assays used. While there are no additional reports describing a C4aR that is distinct from the C3aR, our data do not exclude such a possibility. Through an interaction with the C3aR, human C4a may function as an anaphylatoxin in guinea pigs, but not in mouse or man.

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Exhibit 7

LETTERS

nature biotechnology

Human C5aR knock-in mice facilitate the production and assessment of anti-inflammatory monoclonal antibodies

Hyun Lee[1], David Zahra[1,2], Alexis Vogelzang[1,2], Rebecca Newton[1], Jenny Thatcher[1], Annie Quan[1], Trina So[1], Jörg Zwirner[3], Frank Koentgen[4], Søren B Padkjær[5], Fabienne Mackay[1], Peter L Whitfeld[2] & Charles R Mackay[1,2]

Complement component C5a binds C5a receptor (C5aR) and facilitates leukocyte chemotaxis and release of inflammatory mediators. We used neutrophils from human C5aR knock-in mice, in which the mouse C5aR coding region was replaced with that of human C5aR, to immunize wild-type mice and to generate high-affinity antagonist monoclonal antibodies (mAbs) to human C5aR. These mAbs blocked neutrophil migration to C5a *in vitro* and, at low doses, both prevented and reversed inflammatory arthritis in the murine K/BxN model. Of ~40 mAbs generated to C5aR, all potent inhibitors recognized a small region of the second extracellular loop that seems to be critical for regulation of receptor activity. Human C5aR knock-in mice not only facilitated production of high-affinity mAbs against an important human therapeutic target but were also useful in preclinical validation of the potency of these antagonists. This strategy should be applicable to other important mAb therapeutics.

Chemoattractant receptors, a class of G protein–coupled receptors that facilitate cell migration[1,2], have attracted considerable interest, because blocking these receptors or their ligands ameliorates various inflammatory conditions in animal models[2–4]. However, development of suitable small-molecule antagonists for many chemoattractant receptors has been problematic. Recently, mAbs have been used to antagonize chemoattractant receptors and to identify important regions for ligand binding[5]. The receptor for C5a plays a critical role in numerous inflammatory conditions[6–9]. C5a is a member of the complement cascade, an important mechanism for host defense against bacteria. Increased complement activation and excessive production of C5a have been implicated in the pathogenesis of several inflammatory diseases, and considerable effort has gone into developing C5aR antagonists, including organic small molecules and peptide antagonists[10].

mAbs constitute a rapidly growing class of therapeutics, partly because of their predictable pharmacokinetic properties, their high success rate in the clinic[11,12] and their ability to antagonize interactions between large proteins such as a receptor and its protein ligand. However, poor cross-reactivity of certain mAbs between humans and rodents complicates use of the same drug both for preclinical studies involving rodents as well as for human therapies. Usually, 'surrogate' mAbs are used to examine efficacy or safety in models of disease, and results with such antibodies in mice or even primates may not always equate with the properties of the same or a different drug used in human clinical trials, as noted recently with mAbs to CD28 (anti-CD28 mAbs)[13]. We reasoned that the availability of human C5aR knock-in mice might provide an effective means to validate this receptor in mouse models of disease, using reagents that could be developed for use in clinical trials. Additionally, human C5aR knock-in mice could be a convenient means of generating very high-affinity antagonistic mAbs to human C5aR, through immunization of wild-type mice with neutrophils from human C5aR knock-in mice.

We raised mAbs to human C5aR, first by using a well-tried approach for chemoattractant receptors[5,14,15]. Mice were immunized with L1.2 cells (a mouse B-cell lymphoma line) with high expression of human C5aR (~80,000 receptors per cell). Five fusions yielded several mAbs that reacted specifically with human C5aR transfectants but not with transfectants expressing other closely related chemoattractant receptors, such as CXCR1, CXCR2 or the other C5a-binding receptor, C5L2 (ref. 16) (**Fig. 1a**). Several of the mAbs inhibited both binding of ^{125}I-labeled human C5a to human C5aR transfectants as well as chemotaxis of human neutrophils to C5a *in vitro* (see **Supplementary Fig. 1** online).

In a second approach to develop potent anti-human C5aR mAbs, we reasoned that wild-type mice immunized with neutrophils from human C5aR knock-in mice should mount a focused response to human C5aR, because it will be the only foreign antigen and is expressed highly on neutrophils (as many as 200,000 molecules per cell)[1]. 'Humanized' C5aR mice were generated by targeted-homologous recombination at the mouse C5aR gene (C5aR1). Simultaneous

[1]Immunology and Inflammation Department, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, NSW 2010, Australia. [2]G2 Inflammation Pty. Ltd., 384 Victoria Street, Darlinghurst, NSW 2010, Australia. [3]Department of Immunology, Georg-August-University Göttingen, Kreuzbergring 57, D-37075 Göttingen, Germany. [4]Ozgene Pty. Ltd., P.O. Box 1128 Bentley DC, WA 6983 Australia. [5]Novo Nordisk A/S, Novo Nordisk Park, DK-2760 Måløv, Denmark. Correspondence should be addressed to C.R.M. (c.mackay@garvan.org.au).

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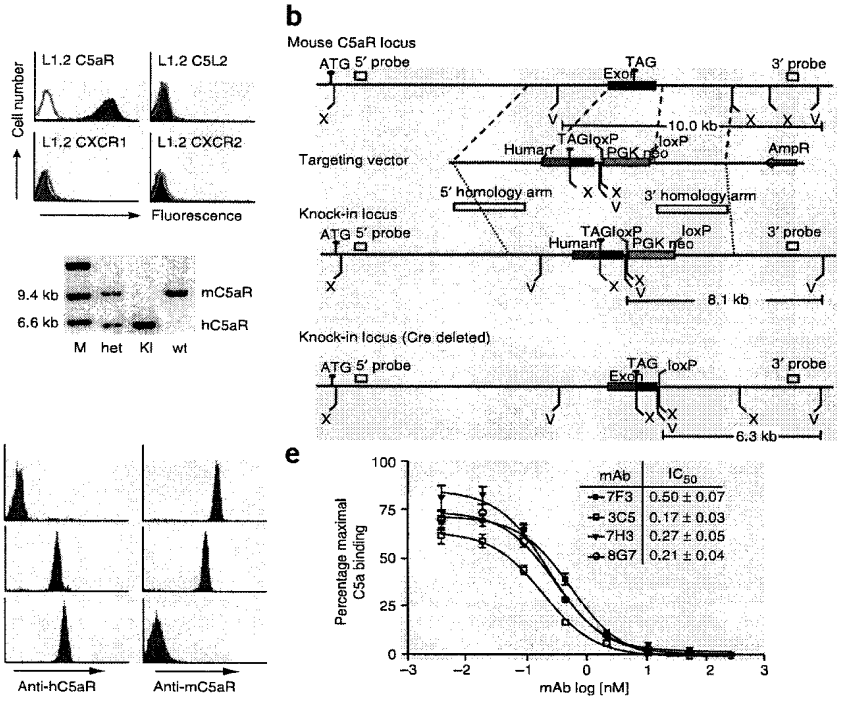

Figure 1 Generation of human C5aR knock-in mice and anti-human C5aR mAbs. (**a**) L1.2 transfectants expressing high levels of C5aR stained with a representative anti-C5aR mAb 7F3 (filled histogram) or IgG2a isotype control antibody (open histogram). mAb 7F3 stained human C5aR transfectants but not L1.2 cells transfected with CXCR1, CXCR2 or the second C5a-binding receptor C5L2. (**b**) Map of the *C5aR* locus in wild-type mice, and in human C5aR knock-in mice, and details of the targeting vector used to create human C5aR knock-in mice. X, *Xba*I; V, *Eco*RV. (**c**) Southern blot of *Eco*RV-digested genomic DNA from the tails of mice from a cross between heterozygous human C5aR knock-in mice (*hC5aR1*$^{+/-}$). M, marker; KI, *hC5aR1*$^{+/+}$; het, *hC5aR1*$^{+/-}$; wt, wild-type mice. (**d**) Expression of C5aR on neutrophils from KI, het and wt mice. Neutrophils were stained with fluorescein isothiocyanate–conjugated anti-human C5aR mAb 7F3 or anti-mouse C5aR mAb 20/70. (**e**) Anti-C5aR mAbs showed subnanomolar IC_{50} values. Antibodies generated using *hC5aR1*$^{+/+}$ mice neutrophils (3C5, 7H3 and 8G7) showed IC_{50} values two- to threefold lower than the best mAb generated using L1.2/hC5aR transfectants (7F3). IC_{50} values were determined from three or four independent competitive ^{125}I-C5a ligand-binding experiments. Data are mean ± s.e.m.

deletion of the endogenous mouse C5aR coding sequence and its replacement with human C5aR coding sequence was achieved by transfecting mouse embryonic stem cells with the targeting construct (**Fig. 1b**). Mice homozygous for the human C5aR transgene (hC5aR1$^{+/+}$) were identified by Southern blot analysis (**Fig. 1c**). Neutrophils from these mice had very high expression of hC5aR, as judged by flow cytometry using anti-human C5aR mAb 7F3, whereas neutrophils from wild-type mice were not stained by 7F3 but were stained intensely by the anti-mouse C5aR mAb 20/70 (ref. 17) (**Fig. 1d**). Human and mouse C5aRs share only 65% homology[18], but importantly for the development of human C5aR knock-in mice, mouse and human C5a bind human C5aR with similar affinities (unpublished observations). Correspondingly, human C5aR was functional in mice, in that neutrophils from hC5aR1$^{+/+}$ mice migrated in response to both human and mouse C5a, *in vitro*, in a similar fashion (data not shown).

After immunization of wild-type mice with neutrophils from hC5aR1$^{+/+}$ mice, we generated numerous human C5aR-specific mAbs. Ligand-binding assays revealed that many of these mAbs showed better inhibition of ^{125}I-labeled C5a binding to human neutrophils than did our best anti-human C5aR mAb generated using transfectants (7F3). The most potent inhibitor, mAb 3C5, had an IC_{50} of 171 pM, compared with an IC_{50} for mAb 7F3 of 503 pM (**Fig. 1e** and **Supplementary Fig. 1b** online). The anti-human C5aR mAbs generated by immunization of wild-type mice with neutrophils from hC5aR1$^{+/+}$ mice were all distinct, in that the amino acid sequences of the heavy-chain variable regions differed, indicating origins from separate clones (data not shown). In human neutrophils, anti-human C5aR mAbs 3C5 and 7F3 blocked calcium flux induced by C5a but not other chemoattractants such as IL-8, fMLP or SDF-1α (see **Supplementary Fig. 2** online). Neither 7F3 nor 3C5 showed agonistic activity in human neutrophils, as measured by calcium flux assays (data not shown). The generation of numerous high-quality and high-affinity anti-human C5aR mAbs allowed us to accurately assess C5aR expression on human leukocytes. C5aR was expressed at high levels on neutrophils, eosinophils, basophils and monocytes, but was absent from all subsets of naive, memory or effector T cells, and from most B cells (see **Supplementary Fig. 3a** online). This protein expression profile was also confirmed at the RNA transcript level, using Affymetrix Genechip profiling (see **Supplementary Fig. 3b** online), establishing C5aR as a receptor on innate and effector leukocytes but not T cells.

The use of cells expressing the entire C5aR allowed us to generate anti-human C5aR mAbs recognizing, potentially, any of the extracellular domains, as well as C5aR epitopes dependent on conformation or comprising more than one domain. The identification of regions on C5aR necessary for ligand binding or function is an important consideration for drug development. Increasingly, mAbs are proving to be useful for identifying these regions[15]. Several studies have implicated the N terminus of C5aR as an essential region for ligand binding[19–21]. We therefore determined the critical epitopes of human C5aR recognized by all of the blocking mAbs. Because these antibodies recognize human but not mouse C5aR, we constructed a panel of human/mouse C5aR chimeric receptors (**Fig. 2a**). Single or multiple extracellular domains of human C5aR were sequentially replaced by the homologous region from mouse C5aR using an overlapping extension polymerase chain reaction (PCR) method[22]. Chimeric receptors were expressed in mouse L1.2 cells, and mAb reactivity was determined by flow cytometry (**Fig. 2a**). Of the 24 antibodies tested, most bound to either the N terminus or the second extracellular loop of human C5aR. However, without exception, all antibodies with the most potent C5aR blocking activity bound to the second extracellular loop (**Fig. 2b**). To define the precise contact residues of the most potent blocking antibodies on the C5aR second extracellular loop, we performed peptide scan analyses using a set of 12-mer peptides, each overlapping by 11 amino acids, covering the second extracellular loop of human C5aR. mAbs 3C5 and 7F3 showed

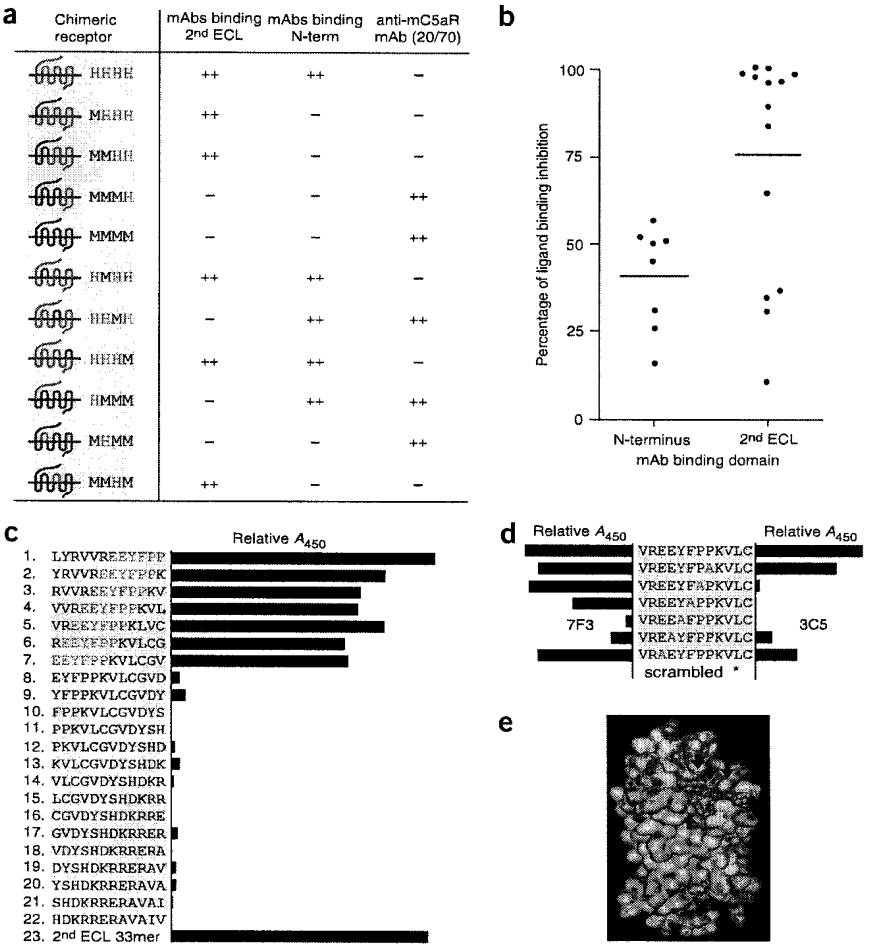

Figure 2 Potent antagonistic anti-C5aR mAbs map to a specific region on the C5aR second extracellular loop. (**a**) Binding of C5aR-specific mAbs to the chimeric human/mouse C5aR receptors. Chimeric receptors are shown schematically (regions derived from human C5aR are shown in red and from mouse C5aR in black). The origin of the four extracellular domains is designated by four-letter code (HHHH is wild-type human C5aR, mHHH has mouse N-terminal extracellular domain and human first, second, and third extracellular loops, etc.). All anti-human C5aR mAbs showed distinct, domain-restricted binding profiles, binding either to receptors containing the human C5aR N terminus or the second extracellular loop. The anti-mouse C5aR mAb 20/70 binds to chimeric receptors containing the mouse C5aR second extracellular loop. (**b**) Dot plot showing the degree to which each individual anti-human C5aR mAb inhibited C5a binding to human neutrophils. mAbs are grouped according to the receptor domain they recognized. (**c**) Mapping of antibody-binding sites on human C5aR second extracellular loop by peptide ELISA. mAbs 7F3 and 3C5 bound to all of the overlapping peptides from human C5aR second extracellular loop containing the sequence ^{179}EEYFPP^{184}. (**d**) Mapping antibody contact residues using alanine mutants of 12-mer peptides identified the critical residues on human C5aR recognized by mAbs 7F3 and 3C5. A nonspecific scrambled peptide was used as a control (asterisk, bottom peptide). (**e**) Molecular representation of the surface of human C5aR with the binding site for 7F3 and 3C5 indicated by arrows.

strong binding to the seven peptides containing the common hexapeptide sequence ^{179}EEYFPP^{184} (**Fig. 2c**). Mutagenesis studies[23] revealed that this region of human C5aR was critical for the regulation of receptor activity, possibly by stabilizing ligand-dependent activation, or regulating the activity of the receptor. Amino acid substitutions in the YFPP motif rendered C5aR constitutively active[23], presumably mimicking conformational changes that follow C5a binding to this region. The epitope ^{179}EEYFPP^{184} was further studied using alanine substitution of every amino acid spanning this peptide region. **Figure 2d** shows that amino acids Glu179, Glu180, Tyr181 and Phe182 were critical for peptide recognition by mAbs 3C5 and 7F3. The EEYFPP epitope region is shown in a predicted three-dimensional structural model of human C5aR (**Fig. 2e**), generated using the rhodopsin structure as a template, and alignment of conserved C5aR residues.

Transgenic mice that express human molecules provide a convenient means to test therapeutics intended for human use in appropriate animal models. C5aR is essential for the pathogenesis of inflammatory arthritis in mice, because C5aR-deficient mice are protected from arthritis induced by either anti-glucose 6-phosphate isomerase autoantibodies[8] or type II collagen mAbs[24]. Anti-human C5aR mAbs were tested for their ability to protect or reverse the progression of experimental arthritis in hC5aR1$^{+/+}$ mice. Transfer of serum containing autoantibodies from arthritic K/BxN mice to healthy mice induces a joint-specific inflammatory reaction that mimics the disease that develops spontaneously in K/BxN mice[25]. After K/BxN serum transfer, hC5aR1$^{+/+}$ mice pretreated with control antibody showed typical clinical arthritis with joint swelling and inflammatory infiltrates consisting mostly of neutrophils, whereas mice pretreated with an anti-human C5aR mAb were completely free of inflammation, clinically and histologically (**Fig. 3a,d**). There was no difference between hC5aR1$^{+/+}$ mice and control littermates in the rate of disease development (data not shown). This indicates that the human C5aR was fully functional, in that disease in the K/BxN model is dependent on C5aR (ref. 8). More importantly, when antibody was given 5 d after disease induction there was a complete reversal of inflammation (**Fig. 3b,c**). As little as 1 mg/kg of mAb 3C5 reversed inflammation and provided a complete and sustained inhibition of disease. A similar reversal of inflammation in arthritis models has also been observed with anti-C5 mAbs[8,26], although we saw this effect with very low amounts of anti-C5aR mAbs. mAb 3C5 and other anti-C5aR mAbs raised against human C5aR–expressing mouse neutrophils gave the greatest degree of inhibition of inflammatory arthritis in the K/BxN model, consistent with the higher affinities of these mAbs over mAbs raised against L1.2 cells expressing human C5aR (**Fig. 3c**). The most potent mAb, 3C5 (IgG3), showed no evidence of neutrophil depletion or margination, because neutrophil counts in blood were not affected by mAb treatment (see **Supplementary Fig. 4** online), and mAb 7F3 (IgG2a) showed only a modest reduction at early time points (data

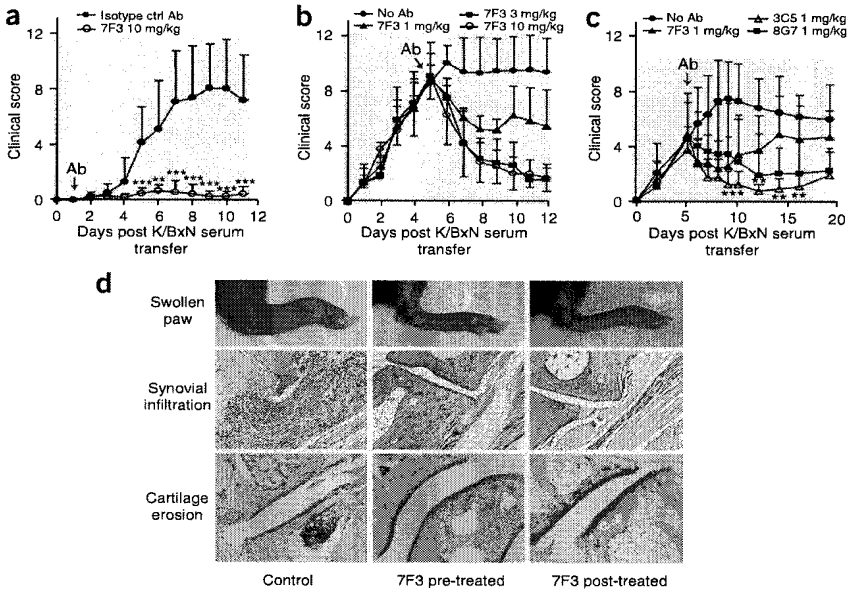

Figure 3 Anti-human C5aR mAbs prevent and reverse K/BxN serum-induced inflammation in human C5aR knock-in mice. (**a**) Preventative treatment: *hC5aR1*$^{+/+}$ mice were injected i.p. with 7F3 or isotype control antibody (each 10 mg/kg in PBS) on days −1 and 1. Clinical score data (maximum possible = 12) are mean ± s.d. ($n = 8$ per group). (**b**) Therapeutic treatment: *hC5aR1*$^{+/+}$ mice were injected i.p. with 7F3 (1, 3 or 10 mg/kg in PBS) once, on day 5 after inflammation had developed. The control group received no antibody. Data are mean clinical score ± s.d. ($n = 3$ per group). (**c**) Comparison of therapeutic efficacy of anti-human C5aR mAbs. *hC5aR1*$^{+/+}$ mice were injected i.p. with 7F3, 3C5 or 8G7 (each 1 mg/kg in PBS) once, on day 5 after inflammation had developed. Data are mean clinical score ± s.d. ($n = 6$ per group). Clinical scores and ankle thickness were measured blind. Statistical analysis compared control groups and anti-C5aR mAb-treated groups each day. $^{***}P < .001$, $^{**}P < .01$, $^{*}P < .05$. (**d**) Representative photographs and sections of hind paws from *hC5aR1*$^{+/+}$ mice treated with 10 mg/kg isotype control antibody (left) or 7F3 (center) in the preventative regimen and treated with 3 mg/kg 7F3 (right) in the therapeutic regimen. Hematoxylin and eosin staining shows cellular infiltration to the joint and severity of histological arthritis. Safranin O staining shows cartilage structure. Original magnification ×100.

not shown). Anti-C5aR mAb therapy in a collagen-induced arthritis model showed similar results to that seen in the K/BxN model (data not shown). mAb 3C5 showed a longer half-life than mAb 7F3 after injection into mice (see **Supplementary Fig. 5** online), which may also account partly for the superior efficacy of mAb 3C5 *in vivo*.

The availability of human C5aR knock-in mice allowed us to generate very high-affinity anti-human C5aR mAbs. Presumably this results from the very focused immune response to human C5aR expressed at high densities on hC5aR1$^{+/+}$ mouse neutrophils. Human C5aR knock-in mice also made possible the evaluation of new therapeutics, designed ultimately for human use. In our studies, anti-human C5aR mAbs were remarkably effective in treating inflammatory arthritis in mice. The relatively low cost of creating humanized knock-in mice and their potential use in studies on safety and efficacy indicate that such mice could well become a standard component of preclinical development. However, in our studies with C5aR we were fortunate that human C5aR bound mouse C5a with high affinity, which eliminated the need to create mice expressing both human C5 and C5aR.

Another important outcome from this study was the development of a mAb that could completely prevent and reverse inflammatory arthritis. Indeed, other than anti-C5 treatment[8,26], we are unaware of any other treatment that does so as completely. Notably, anti-C5aR mAbs showed this effect at doses (1 mg/kg) considerably less than the doses of anti-C5 antibody used to inhibit arthritis in mice (~40 mg/kg)[10,26]. One reason for this may be the high concentration (~180 μg/ml) of C5 that is normally present in blood and tissue fluids. In addition, our mAbs recognize C5aR but not C5L2, the second C5a-binding receptor that provides inhibitory signals upon C5a binding[16]. Thus, some inhibition of the inflammatory response by anti-C5aR mAbs may result from uninterrupted binding of C5a to C5L2. The very rapid reversal of inflammation with anti-C5aR mAb treatment was surprising but probably relates to the critical and ongoing requirement of neutrophils and/or mast cells for perpetuating the disease process. Neutrophil depletion using a depleting mAb also reverses inflammation in the K/BxN model[27], suggesting that the continuous release of proinflammatory mediators by neutrophils is necessary to perpetuate certain inflammatory responses.

METHODS

Animal ethics. All experimental procedures involving mice were carried out according to protocols approved by the Garvan Institute–St. Vincent's Hospital Animal Ethics Committee and the Animal Resources Center–Ozgene Animal Ethics Committee.

Generation of human C5aR knock-in mice. A knockout–knock-in strategy was adopted to construct a transgenic mouse expressing human C5aR, but not mouse C5aR, under the control of the mouse C5aR gene promoter. The targeting vector comprised a 3.5-kilobase region of mouse C57BL/6 genomic DNA upstream of the C5aR gene exon 2, human C5aR gene exon 2 coding sequence, mouse C5aR gene 3′ untranslated region, PGKneo flanked by loxP sites and a 3-kilobase region of mouse genomic DNA downstream of the C5aR gene in the vector pLOz (Ozgene). Genomic DNA fragments were generated using PCR amplification. The vector was transfected into C57BL/6 Bruce4 stem cells[28], and DNA from G418-resistant colonies was screened by Southern blot. *Xba*I- and *Eco*RV-digested DNA was hybridized with 5′ and 3′ probe, respectively, to identify clones with the correct homologous recombination event at both 5′ and 3′ ends. Two embryonic stem clones out of 672 screened were identified as containing the correctly targeted human C5aR sequence. Five chimeric mice were produced from these embryonic stem cells, thus establishing the human C5aR knock-in line. Chimeras were mated with C57BL/6 females. Germline transmission of the human C5aR gene was confirmed by Southern blot of mouse tail genomic DNA (**Fig. 1d**). Mice homozygous for the human C5aR gene (*hC5aR1*$^{+/+}$) were generated, and PCR, Southern blot and flow cytometry confirmed the absence of mouse C5aR. The PGKneo gene flanked by loxP sites was deleted from the knock-in locus using a BL/6 Cre deleter strain.

Neutrophil isolation. Human neutrophils were isolated from the peripheral venous blood of healthy volunteers. Blood samples collected into ethylenediamine tetraacetic acid–coated vacutainers were centrifuged at 400 *g* for 15 min, and then the plasma and buffy coats were removed. After 1% dextran sedimentation for 30 min the white blood cells were pelleted by centrifugation at 300 *g* for 5 min and washed with phosphate-buffered saline (PBS). The cells

were then centrifuged at 500 *g* for 30 min on a cushion of 65% Percoll (density 1.093 g/ml, Amersham Bioscience). After centrifugation, the neutrophils were resuspended in PBS. Mouse neutrophils were isolated from both hind leg femurs by forcing 5 ml Dulbecco's modified Eagle medium (Gibco) with 10% fetal calf serum through the bone with a syringe. Neutrophils were separated by density centrifugation over Ficoll-Paque (Amersham Bioscience). Red blood cells were lysed using hypotonic buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM ethylenediamine tetraacetic acid). Cell viability was determined by trypan blue exclusion, and the neutrophil pellet was resuspended in PBS.

Monoclonal antibody generation. C57BL/6 mice were immunized i.p. with 10^7 transfected L1.2 cells with high expression of human C5aR (ref. 29), six times at 2-week intervals, five i.p. and one i.v. for the last immunization. At 4 d after a final i.v. immunization, the spleen was removed and the cells fused with the SP2/0 cell line using standard procedures. C57BL/6 mice were immunized with 10^6 neutrophils isolated from blood of *hC5aR1*$^{+/+}$ mice, in a similar fashion. Hybridomas were grown in Dulbecco's modified Eagle medium (Gibco) containing 10% Fetalclone (HyClone) and were purified by protein A or G chromatography and concentrated. Buffer was exchanged and endotoxins removed. mAb concentration was determined using a mouse IgG enzyme-linked immunosorbent assay (ELISA) (Roche Diagnostics).

Flow cytometry. To assess reactivity of mAbs against transfected cells or leukocytes, we used indirect immunofluorescence staining and flow cytometry. Cells were washed once with PBS and resuspended in 100 µl PBS containing 2% (wt/vol) BSA and 0.1% (wt/vol) sodium azide (staining buffer), purified antibody, or 50 µl hybridoma culture supernatant. After 20 min at 4 °C, cells were washed twice with staining buffer and resuspended in 50 µl FITC-conjugated affinity-purified $F(ab')_2$ goat anti-mouse IgG (Jackson Immuno-Research Laboratories) diluted 1:200 in staining buffer. After incubating for 20 min at 4 °C, cells were washed twice with staining buffer and analyzed on the FACSCalibur (Becton-Dickinson) to determine the level of surface expression. Propidium iodide staining was used to exclude dead cells.

Binding assays. Human neutrophils were washed and resuspended in binding buffer (50 mM HEPES, pH 7.5, 1 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.5% bovine serum albumin) at 1×10^7 cells/ml. For each binding reaction (in a final volume of 120 µl), 40 µl cell suspension (4×10^5 cells) with an appropriate amount of anti-human C5aR mAb, isotype-matched control mAb or unlabeled human C5a (Sigma) was incubated at 20–25 °C for 15 min. ^{125}I-labeled human C5a (Perkin Elmer) was added at a final concentration of 0.4 nM, and the reactions were incubated at room temperature for 1 h. Cells were then collected and washed thrice with binding buffer containing 150 mM NaCl. Cells were then transferred to Opti plates (Perkin Elmer) with MicroScint 20 scintillation fluid and radioactivity counted using a TopCount (Packard). Each sample was assayed in triplicate.

Construction of cell lines expressing chimeric human/mouse C5aRs. Chimeric human/mouse C5a receptors were constructed using a modified PCR-based overlap extension technique[22]. Briefly, different fragments of the human or mouse C5aR gene were amplified by PCR. Overlapping fragments were combined, denatured and re-annealed and amplified by second round of PCR. Full-length chimeric receptor sequences with appropriate restriction enzyme sites were amplified in a third PCR step and cloned into pcDNA3.1(+) (Invitrogen) for expression. PCR primers (see **Supplementary Table 1** online) were designed according to the human and mouse *C5aR* gene sequences (GenBank accession numbers M62505 and S46665, respectively).

Transfection of expression vectors into L1.2 cells. Mouse L1.2 cells were grown in RPMI 1640 (Gibco) supplemented with 10% bovine calf serum (HyClone), and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions. Transfected cells were used after 1 d.

Epitope analysis with synthetic peptides. Two sets of peptides with N-terminal biotin and spacer GSGS were synthesized in immobilized form on plastic pins (Mimotopes Pty Ltd.). The first set of 22 peptides contained all possible 12-mers from the human C5aR second extracellular loop, each offset by one amino acid (**Fig. 3c**). The second set were 12-mers of the sequence VREEYFPPKVLC, each with one residue substituted by alanine. Peptides were initially reconstituted in 200 µl 60% dimethyl sulfoxide and subsequently diluted in PBS to give a final concentration of 10 µg/ml for direct ELISA. The full (33-mer) amino acid sequence of the human C5aR second extracellular loop was also synthesized as above (peptide 23).

Peptide ELISA. Streptavidin-coated microtiter plates (Nunc) were coated with 10 µg/ml of peptide per well in a volume of 200 µl, and incubated at 4 °C overnight. Plates were washed three times with ELISA wash buffer (0.05% Tween 20 in PBS). mAbs were added at 2.5 µg/ml, and plates were incubated for 3 h at room temperature. Horseradish peroxidase–conjugated rabbit anti-mouse IgG antibody diluted 1:1,000 in ELISA wash buffer was used for detection. Plates were developed using TMB (3,3′,5,5′ tetramethyl benzidine; Sigma) and read at A_{450nm}.

K/BxN inflammatory arthritis model. Serum was collected from K/BxN arthritic mice as described[25]. Experimental arthritis was induced in recipient mice by injecting 150 µl serum i.p. on days 0 and 2, and disease progress was monitored as described[30]. Ankle thickness and clinical scores were determined daily. The clinical score was calculated for each mouse by summing the scores for the four paws: 0, normal joint; 1, mild-to-moderate swelling of the ankle and/or one swollen digit; 2, swollen ankle or swelling in two or more digits; 3, severe swelling along all aspects of paw or all five digits swollen. Anti-human C5aR or isotype control mAbs (1–10 mg/kg in PBS) were injected i.p. on days −1 and 1 (preventative treatment) or day 5 (therapeutic treatment). At day 12 mice were killed and paws collected for histology. Paws were fixed for 48 h in fixing solution (10% vol/vol phosphate-buffered formalin) and decalcified by treatment with 10% vol/vol formic acid in fixing solution for 5 d. Samples were then washed with PBS and embedded in paraffin. Sections of 6 µm thickness were stained with hematoxylin and eosin or Safranin O.

Statistical analysis. The statistical significance of differences between independent control and treatment groups in the K/BxN model was determined using either the Mann-Whitney test or the Kruskal-Wallis test, and post hoc analysis with Dunn's multiple comparison test.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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